The Rat-Trypanosome, Trypanosoma lewisi, in its Relation to the Rat-Flea, Ceratophyllus fasciatus.

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and

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With Plates 36—45 and 24 Text-figures.

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PART I. INTRODUCTORY.

(1) PERSONAL NARRATIVE.

IN this memoir we give a detailed account of investigations which have occupied us intermittently, with many interruptions, during the past five years. When we undertook this task our object in view was to work out as fully as possible the life-history and mode of transmission of a trypanosome, so that in at least one species of these important parasites its relation to the invertebrate host might be as thoroughly known as, for instance, the relation of the malarial parasite to the mosquito, thus furnishing a standard with which the life-histories of other species of

trypanosomes might be compared and contrasted as they become known. How far we have succeeded in our task must be left to our readers to judge.

The species which we selected as the subject of our investigations was Trypanosoma lewisi, the common parasite of rats which is apparently of world-wide distribution. This species offers many advantages for such a study. It is common in London and easily procured when required; its vertebrate host, the rat, is a mammal of small size, the domesticated variety of which lives well and breeds rapidly in confinement, is inoffensive, and is easily handled; its invertebrate host, the rat-flea, is also easy to keep in captivity and is extremely prolific, and it is of a size which, though it increases to some extent the difficulties of manipulation, has a great advantage that the material to be searched and studied microscopically is confined within a small compass[1]; and finally, by no means the least of the advantages of working with T. lewisi is the fact that it is non-pathogenic to its natural host and cannot live at all in human blood.

Since there is no difficulty whatever in obtaining the vertebrate host in abundance, either in the clean (i. e. non-infected) or infected condition, our first care was to obtain a stock of the invertebrate host, the flea. This we succeeded in doing from rats trapped in the open near Mr. Gurney's Laboratory at Sutton Broad, Norfolk. Fifty specimens of Ceratophyllus fasciatus were obtained in this way in the autumn of 1908, and were kindly identified for us by the Hon. N. C. Rothschild, and with these fleas a breeding-cage was stocked and a flea-farm started. The cages used were of the type used by the Plague Commission, as figured in the 'Journal of Hygiene,' vol. vi, pl. iv. A rat was kept in the cage to feed the fleas, and they were left to themselves. Early in 1909 one of us (E. A. M.) went to Rovigno for some

[1] An advantage which those will appreciate who have had practical experience of searching for trypanosomes through many centimeters of the digestive tract of the tsetse-fly, for instance.

three months, during which time the fleas were left to breed under the care of an assistant, whose duties consisted of attending to the rat and replacing it if it fell ill or died. When the cage was examined after Easter it was found to be swarming with fleas, and our work began in May, 1909. We have worked ever since then with the fertile progeny of the original fifty fleas from Norfolk, and have never added further to our stock from without. The fleas breed so fast that it is often necessary to keep their numbers down, otherwise they take too much blood from the rat and affect its health. Fresh breeding-cages have also been started, and during the greater part of the time that we have been at work we have kept two cages constantly going, one in which the fleas are fed always on a clean healthy rat, and another in which an infected rat is always kept. We shall refer to these two cages as the non-infected and the infected breeding-cages respectively. As will be shown below, the stock of fleas with which we have worked all along was fortunately quite free from any natural infection with leptomonad or other flagellate parasites. Thus we have been saved from a fertile source of confusion and error, since we can be quite certain that any flagellates found in our fleas are stages of *T. lewisi* and nothing else.

Although we cannot claim that in our work we have solved completely every problem presented by the transmission of the trypanosome and its development in the flea—a result which probably no man could achieve in a life-time—we think it now fitting that we should publish such results as we have obtained, after having done as much as we were able to do in the time and under the circumstances. We claim at least that we have not jumped to our conclusions; our note-books contain not only the records of many experiments, but also of the dissection and examination of over 1,600 fleas, and we have over 700 drawings of stages of the development of the trypanosome, from which those given in this memoir are a selection. It would, indeed, have been easier for us to have written a plausible and apparently complete account of

the development of T. lewisi, full of positive statements, after one year of our work than it is after five years, during which we have been forced by the logic of facts to abandon or modify many of our earlier conclusions or beliefs.

It is our pleasant duty at this point to express our thanks to those of our friends and colleagues to whom we are indebted for assistance. To Dr. Woodcock and Miss Robertson we are grateful for much advice, friendly criticism, and valuable suggestions. Our work could not have been carried out, certainly not in the time at all events, without the assistance of Miss Rhodes, who has not only drawn all the illustrations with a skill to which it is quite unnecessary for us to draw the reader's attention (since the figures speak for themselves), but has also relieved us of a large part of the wearisome drudgery of searching through the microscopic preparations. Mr. George Kauffmann has been most helpful in every part of the investigation, not only assisting in making preparations, examining rats, and other similar duties, but more especially in carrying out intelligently and enthusiastically all the details of the experiments, in which his extraordinary skill and resourcefulness in controlling the wayward flea were invaluable. Dr. D. J. Reid has given us the benefit of his skill and experience in microphotography. From our colleagues of the Lister Institute, Dr. C. J. Martin, the Director, and Mr. Bacot, who have been themselves engaged in studying the transmission of plague by fleas, we have had many valuable hints and help in various ways. To each and all of these we desire to express our cordial thanks and gratitude.

(2) Notes on the Flea, Ceratophyllus fasciatus.

(a) Anatomy. Methods of Dissection.

The fleas collected for dissection and examination were thrown, or allowed to hop, on to the surface of a small

quantity of salt-citrate solution[1] placed in a suitable glass capsule. The fleas are quite helpless on the surface of the liquid, and each flea that it is required to dissect can be picked off the surface of the liquid and transferred to a small drop of the same solution on a slide for further operation.

The examinations of the fleas were usually conducted by both of us acting in concert. One of us worked with the dissecting-microscope, extracted the parts of the flea required, placed them on slides, covered them with glass slips, and handed them to the other, who proceeded to search them carefully through under a microscope, using dry lenses of fairly high magnification (Zeiss D or apochromatic 4 mm.). In some cases one of us worked entirely alone, but it is difficult for one person to carry out satisfactorily both the dissection and examination of the flea; the various parts of the digestive tract often require prolonged and careful searching to find the flagellates, and if the operator be working single-handed, one preparation may dry up while he is searching through another.

For the dissection of the flea[2] the following apparatus was used: A pair of fine needles mounted in wooden handles, a fine pair of forceps, and a dissecting-microscope, besides slides and coverslips. The needles used were sharpened on a hone, one to a sharp point, the other to a flat, chisel-like edge with rounded corners. The pointed needle was the more useful for holding, the flat-edged needle

[1] Made up as recommended by Laveran and Mesnil, namely: 1 grm. of sodium citrate and 1 grm. of sodium chloride dissolved in 200 cc. of distilled water. This mixture appears to be most favourable for the examination of living trypanosomes.

[2] If an operation can be properly called dissection which consists in treating the flea as the Thracian women are said to have treated Orpheus: "Discerptum latos juvenem sparsere per agros" (i. e. fields of the microscope, in this case). It need hardly be said that our object was not to study the anatomy of the flea, but to extract from its body those organs which might possibly harbour developmental stages of the trypanosome.

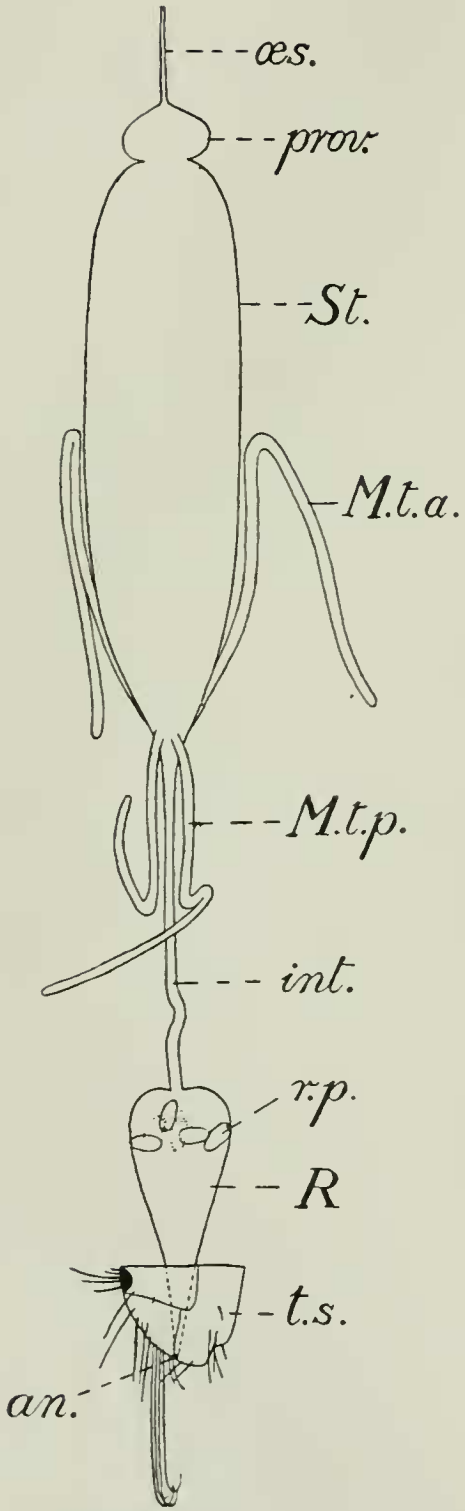
for cutting. The dissecting-microscope used was a Zeiss binocular, with No. 4 eyepieces and the paired objectives F^{55}. It is also necessary for the dissector to have at hand an ordinary microscope armed with a low power, since it is often difficult to distinguish the minute organs of the flea under the dissecting-microscope; the intestine, for instance, if severed from its connections might easily be confounded with a portion of a Malpighian tubule.

In the following paragraphs is described the method of procedure for making what may be considered an exhaustive examination of a flea for trypanosomes; it was not always necessary, however, to attempt so much, nor is it claimed that the entire operation was always successfully carried out, since both our knowledge of the flea's anatomy, and our skill in extracting the organs required, advanced considerably during the progress of our investigations.

The flea, as stated above, is picked up with a fine pair of forceps, holding it by its head, and placed on a slide (slide 1) in a drop of salt-citrate solution. The first operation is to cut off the head, which is not always easy if the flea be a lively one, in which case it is best to asphyxiate or drown the flea partially by holding it under water with the forceps for a short time. To decapitate the flea, hold it still by pressing the pointed needle across the thorax, and with the flat-edged needle cut across the head in the region of the eyes. The severed head may then be removed to another slide (slide 2), covered with a cover-glass, and the contents of the proboscis examined; but as the proboscis was never found to contain trypanosomes we ceased to trouble about it in our later studies.

It is frequently the case that the flea has its rectum filled with fæces or with partially digested blood, and when this is so it happens commonly that the rectum empties itself by a violent contraction at the instant that the head is severed (sometimes also eggs are extruded); or if the evacuation does not take place at this point in the proceedings, it is very difficult to avoid squeezing out the contents of the distended

TEXT-FIG. 1.



Digestive tract of a female flea, dissected out and drawn with the camera lucida at a magnification of 60, reduced in the reproduction to 40. The anterior part of the dissection is seen in ventral view; the rectum and its surroundings in side view. *æs.* Œsophagus. *prov.* Proventriculus. *St.* Stomach. *Mt. a.* Malpighian tubule of the anterior pair; that on the left side of the stomach is shown in its normal position, that on the right has its distal limb pulled out and away from the stomach. *Mt. p.* Malpighian tubule of the posterior pair. *int.* Intestine. *r. p.* The six rectal papillæ. *R.* The rectum. *t. s.* Terminal segments. *an.* The anus.

rectum during the subsequent operation of opening the abdomen. In cases where fæces are thus extruded the body of the flea is removed at once to another slide (slide 3), and the fæces left on slide 1 are covered with a slip and examined.

Through the integument of the flea the stomach can be seen lying ventrally in the anterior $\frac{2}{3}$ of the abdomen, and often the rectum can be seen at the hinder end in the dorsal region. The ventral posterior and dorsal anterior part of the abdomen is seen to be occupied by a whitish mass, most conspicuous in the female, and consisting chiefly of the reproductive organs.

The next stage in the proceedings is to open the body of the flea. This is done near the hinder end, at about the level of the fourth or fifth tergite of the abdomen. The body is held still with the pointed needle, with which the thoracic region is pressed down or speared, and with the flat-edged needle the body-wall is cut through dorsally and ventrally in the region indicated, and the hindermost segments of the abdomen gently detached in such a way as to separate the integumental portions without rupturing or tearing the internal organs. It is especially important, if it be desired to examine the contents of the body-cavity, that the digestive tract should not be in any way torn or punctured. By holding the anterior part of the body and pulling gently on the detached hinder part, the gut can be stretched out and seen in nearly its full length; the stomach, usually containing a greater or less amount of more or less digested blood, is seen projecting from the anterior part of the body, the rectum is contained in the detached hinder part, and stretching between the two is the intestine like a delicate white filament, exposed in its whole length, but more or less obscured by the fat-body, Malpighian tubules, and generative organs, especially by the large ovaries in the female; these organs render the female flea much more difficult to dissect, in spite of its larger size, than the less-encumbered male. The generative organs and as much as possible of the fat-

body are now pulled out on to the slide and cut off from the body, care being taken not to injure the gut. The carcase of the flea, with the hinder part hanging on by the still intact intestine, is now removed to another slide (slide 4), and the extracted contents of the body-cavity on slide 3 can be covered with a slip and passed on for examination; but so far as stages of T. lewisi are concerned, it is superfluous to do so, since they are never found in the body-cavity unless the gut has been punctured or ruptured.

The next step is to divide the digestive tract into two parts, thereby severing completely the hinder part of the body from the fore-part. This is done at the point at which the Malpighian tubules are given off at the junction of the stomach and intestine, the region which represents the transition from the mid-gut, lined by endoderm, to the hind-gut or proctodæum, lined by ectoderm. The Malpighian tubules are four in number in the flea; two of them run forward a short way on the wall of the stomach right and left, attached to it by fine tracheal tubes, and then turn backwards again with a sharp, elbow-like bend towards the dorsal side of the body; the other two tubules run backwards parallel to the intestine and alongside of it towards the hinder end of the body. The posterior pair of the tubules are also bent on themselves towards their distal extremities, but not so regularly as the anterior pair. The gut is cut across with the flat-edged needle at the point of origin of the tubules, and if this be performed accurately one pair of tubules (the anterior pair) remains attached to the stomach, the other pair to the intestine; sometimes, however, all four tubules remain attached to one or other of these organs. The hinder part of the body, with the intestine and rectum, is now removed to another slide (slide 5). The stomach is then pulled backwards out of the anterior part of the body on slide 4, and with it come out also, continuous with its anterior termination, the proventriculus and the œsophagus, these two parts representing the embryonic stomodæum, lined by ectoderm, while the stomach represents the whole of the embryonic mid-

gut. The proventriculus is lined by a thick chitinous cuticle prolonged into stiff, curved, pointed spines, densely planted and forming, apparently, a straining apparatus; it is approximately globular in form and usually contains blood. The œsophagus is a delicate tube, its walls composed of the chitinous cuticle internally and a delicate network of muscles externally; it generally performs active movements, twisting from side to side, when freshly extracted.

The two pairs of salivary glands are situated in the anterior region of the abdomen right and left of the stomach. Each gland has the form of a simple oval pouch, the wall of which is composed of a single layer of large cells with very large nuclei. From each gland comes off a duct, which, after running a short distance, unites with the similar duct of the other gland of the same pair. (In one instance we have seen the two glands of one side of the body fused into one, but with their ducts quite separate; on the other side of the body there was a pair of distinct glands in their normal relations). The common duct of each pair of glands then passes forwards alongside the gut through the thorax into the head, where it meets and joins with the corresponding duct from the other side of the body. The common salivary duct then runs a short distance and opens into the proboscis, doubtless on the hypopharynx as in other insects. The salivary ducts are recognisable at once under the microscope by their trachea-like structure, being lined by a thick cuticle which has ring-like thickenings; the rings are, however, somewhat irregular and easily distinguishable from the very even and regular spiral thickening of the wall of a tracheal tubule. Externally to the cuticular lining the tubule is covered by an investing layer of protoplasm, of uneven thickness in different parts and containing fairly large nuclei at irregular intervals. The ring-like thickenings of the cuticular lining become less marked as the ducts approach their point of junction, and cease altogether before they unite; the cuticular lining being quite smooth in the common duct and for short distances in the paired ducts.

Not infrequently the salivary glands come out with the stomach when it is pulled out; more usually, however, they do not do so, but remain *in situ*. In such cases the anterior part of the body is removed to another slide (slide 6), and the stomach, left on slide 4, is teased up, covered, and handed on for examination.

Now the dissection of the hinder part of the body, on slide 5, is proceeded with, in order to extract and separate the intestine and rectum. The rectum, situated dorsally to the accessory reproductive apparatus, penis or receptaculum seminis, is a fairly large pear-shaped organ, the stalk of the pear terminating in the anus. The slender intestine joins the rectum at its broad end, and in this region are situated the six conspicuous rectal papillæ, remarkable and very characteristic structures, the presence of a single one of which makes it easy to recognise even a small fragment of the rectum. Behind the papillæ the rectum has a thin wall, to which the crithidial stage of the trypanosome, when present, is usually found attached, sometimes in vast numbers. In its anterior part, the region of the papillæ, the rectum has only circular muscle bands, between which are wide interspaces. In the hinder region, behind the papillæ, there are both circular and longitudinal muscle-bands; the latter can be traced forward to just behind the papillæ, at which point each band becomes rapidly narrowed to a tendon-like fibre, and at the same time the striations of the muscle disappear. The tendinous continuations can be traced forwards, in the living condition, for some distance, but we have not made out the exact points of their insertion.

The intestine is characterised by a continuous coat of ring-like muscle-bands, with interspaces, arranged very regularly external to the epithelium. When the edge of the intestine is focussed under the microscope, the layer of circularly-disposed muscle-fibres is seen in optical transverse section like a string of beads. The intestine is frequently seen to be performing active peristaltic movements, and it may be thicker in some parts than in others, owing to the contraction of the muscles.

The rectum must be dissected carefully out of the hinder part of the body, so that it remains on the slide, free from all the adjacent organs or chitinous plates of the integument. The easiest way to do this is to make an obliquely longitudinal cut with the flat-edged needle so as to sever the ventral-anterior half of the hindmost segments, together with the genitalia, from the dorsal-posterior half containing the rectum and anus. The genitalia can then be removed and the rectum extracted without much difficulty. It requires some care to separate it from the anus without injuring it. When this has been accomplished, all unnecessary débris is cleared away. If it be desired to make separate examinations of the intestine and rectum, the intestine is cut off as close as possible to its junction with the rectum. To effect this it is best to spear the rectum with the pointed needle and make the cut with the flat-edged needle; or the operation of cutting off the intestine may be performed before the rectum has been dissected out from the hinder part of the body. In either case, the intestine is removed to another slide (slide 7), and both rectum and intestine, on their respective slides (5 and 7) are teased up, covered, and passed on for examination. It is not difficult to tear the rectum into several pieces with the needles, but it is not so easy to tease up the intestine; it is too slender to make sure of splitting it lengthways, except by good luck and more or less accidentally, and it is necessary as a rule to content oneself with cutting it transversely into two or three short pieces, the contents of which are generally squeezed out during the process.

Finally there remain the salivary glands, on slide 6, in the portion of the carcase consisting of the thorax and fore-part of the abdomen from which the gut has been extracted. The salivary glands, as has been stated above, are lodged in the fore-part of the abdomen beside the stomach, and it is generally by no means difficult to extract them when the stomach has been removed. To do this it is best first to spear the thorax with the pointed needle, then insert the flat-edged needle into the abdominal cavity from behind, and rake

out gently the contents of the abdomen. The salivary glands sometimes come out fairly clean, but more often they are embedded in fat-body, tracheæ, etc., from which they must be carefully freed as much as possible. In such cases they are sometimes a little difficult to detect under the dissecting microscope, but their position may be traced by their long, thread-like ducts. They are much smaller in the male flea than in the female. Another method which sometimes succeeds better in extracting the glands is to pull on the integument of the thorax with one needle and on that of the abdomen with the other. The body-wall then often tears across at the junction of the thorax and abdomen, and the salivary ducts are seen at once stretched out between the two. By continuing to pull the thorax forwards, the glands may be pulled out of the abdominal cavity and are seen hanging on to the back of the thorax, from which it is not difficult to detach them. By this method the glands may often be obtained very clean and free from encumbering fat and other tissue. When the glands have been extracted, other débris is cleared away and the coverslip is put on. The glands are very soft and are crushed immediately by the weight of a coverslip if there is no other tissue under it; but for examination of their contents this is not a disadvantage.

In the foregoing paragraphs we have given a detailed account of a full examination of the flea, such as we practised in the earlier periods of our investigation. But when it became evident to us that the trypanosome, during its development in the flea, never strays beyond the limits of the digestive tract proper, we were able greatly to curtail the ritual of the examination and to omit entirely the proboscis, body-cavity, and salivary glands. It is also unnecessary, as a rule, to separate the intestine and rectum in the dissection. Consequently, our later examinations were reduced to (1) the excluded fæces, if any, on the slide on which the flea was decapitated; (2) the stomach, on a second slide; and (3) the rectum and intestine, on a third.

It was no part of our task to make a special and detailed study of the

anatomy of the flea, but a few points observed by us incidentally in our dissections may be noted briefly here.

The nervous system, of which some beautiful dissections were made in this laboratory by Major Christophers, I.M.S., consists, as in insects generally, of (1) the brain or supra-œsophageal ganglion-complex, sending off the peri-œsophageal connectives which pass on either side of the œsophagus to connect with the foremost of (2) the three large thoracic ganglia, joined by connectives to form a series which passes on into (3) the abdominal chain of ganglia. It is a very difficult operation to dissect out the brain and the first two thoracic ganglia, but it happens very frequently that in the ordinary dissections of the flea the third thoracic ganglion and the abdominal chain of ganglia are exposed entire and in continuity. It is then seen that the abdominal chain consists of a series of small ganglia terminated posteriorly by a larger ganglion; and further that in the male there are seven smaller ganglia, in the female only six, in the abdominal chain. The larger hindmost ganglion, from which nerves are sent off to the genitalia and rectum, evidently represents a fusion of several ganglia equivalent to the more anterior smaller ganglia. Consequently it is seen that the concentration and fusion of ganglia at the hinder extremity of the ventral chain has proceeded a step further in the female than in the male.

The genitalia consist, in the male, of a conspicuous pair of testes, situated dorsally in the abdomen, and a pair of filamentous glands (prostates?) not unlike Malpighian tubules at first sight, but of slightly smaller calibre, and differing entirely in histological structure. There is no separate seminal vesicle, but each testis is a tightly convoluted tubule, the lower end of which is dilated to contain the ripe spermatozoa. Ducts from the testes and prostates unite to form a median Ductus ejaculatorius, which opens into a large penis of very complicated structure. In the female the two ovaries occupy practically the same position as the testes, but take up much more space and extend forwards to the most anterior limits of the abdomen. Each ovary consists usually of four egg-tubes or ovarioles, but in one specimen that we have mounted as a permanent preparation there are five ovarioles on each side. The ducts of the ovarioles unite to form the paired oviducts, and these unite in their turn to form the common oviduct. Ventral to the common oviduct lies the unpaired receptaculum seminis, consisting of a brown, chitinous capsule of a peculiar shape. The main body of the capsule is spherical, but gives off a curved, horn-shaped diverticulum, ending blindly. The horn-shaped portion has its concave curve turned towards, and connected by striped muscles with, the spherical part of the capsule. A slender duct of great length, and much convoluted near its origin, arises from the spherical part of the capsule, and runs back to open probably into the distal extremity of the oviduct or into the

genital vestibule. The spherical part of the capsule and duct of the receptaculum are surrounded with unicellular glands, thickly clustered round the capsule and the convoluted portion of the duct, but thinning out and becoming smaller towards the distal end of the duct. The receptaculum, dissected out, stained and mounted for the microscope, is a singularly beautiful object. It usually contains a dense mass of spermatozoa.

The heart is frequently seen in dissections at the hinder end of the body as a delicate filament, which by its own contractions twists and lashes itself about. Under the microscope it appears a delicate tube, beset towards the hinder end by the pericardial cells which are attached to it on either side, right and left, and are crowded together towards the hinder end, but occur more sparingly towards the middle region and are wanting in the anterior third of the heart. The ostia appear to be confined to the posterior region of the heart, but we have not made out their exact number or arrangement. For the pericardial cells, see Minchin (1910).

(*b*) Notes on the Parasites of the Fleas.

In a former publication one of us (E. A. M., 1910) has described some parasites found in our stock of fleas. The most important was a form to which the name Malpighiella refringens was given, occurring, as the generic name implies, in the Malpighian tubules of the flea. Since that time this infection seems to have died out entirely in our fleas, and we have not seen any Malpighiella in the fleas dissected by us for the last three years or more. Why this parasite should have died out in our fleas it is impossible to say, but it may be remarked that no conditions could possibly be more favourable for contaminative infection from flea to flea (whether from adult to adult, or larva to larva, or adult to larva, or vice versa) than those in our breeding cages, where vast numbers of fleas in all stages of development are herded together in a confined space. Consequently the disappearance of Malpighiella in our cages rather indicates that the fleas do not acquire infection with this parasite by the contaminative method.

In the publication referred to, numerous yeast-like bodies were described and figured from the digestive tract of the flea. Since then we have found organisms of this kind abundantly in smears of the salivary glands (text-fig. 24, p. 642).

In the larvæ of fleas that we have dissected and examined from our cages we have found the gregarine Agrippina bona (Strickland, 1912).

The cysticercoids of tapeworms are found not infrequently in the fleas. Nicoll and Minchin (1911) described two species of cysticercoids

from our fleas, representing Hymenolepis diminuta and another species of the same genus. We have found the same two species frequently, and also have in our possession specimens of a third species not identified. The cysticercoids appear sporadically, and are sometimes quite common for a period, and then are not found again for a long time. This uncertainty in their occurrence is quite intelligible, since their appearance must be caused by the introduction into the cage of a rat infected with tape-worms, which doubtless infects a large number of the larvæ that later become adult fleas.

The point upon which we wish to lay special stress is the absence in our stock of fleas of any flagellate parasites, and more especially of the leptomonad described by Swingle (1911) under the name Herpetomonas (Leptomonas) pattoni. We have been at great pains to convince ourselves upon this point. In the first place we dissected at various times about eighty[1] fleas from the non-infected breeding-cage without finding any flagellates of any kind in them, while flagellate parasites occur in a very large percentage of those known to have fed upon infected rats, though not in all, since the trypanosome often fails to establish itself in the flea, and even when the insect has been fed on a rat with trypanosomes swarming in the blood, they often disappear completely from the digestive tract of the flea within twenty-four hours of its having fed.

We give here three tables (A, B (1), and B (2)) showing the results of dissections of fleas from our stock which had been put upon infected rats, and so had had the chance of acquiring an infection of T. lewisi. Fleas do not always feed, however, when given the opportunity to do so, especially in cold weather,[2] and if the fleas are dissected and examined within twenty-four hours after having been put on the rat (the fleas in all cases having been kept hungry for

[1] As a matter of fact we dissected far more than this, all with negative results, but we have not kept exact records of more than seventy-nine.

[2] Note especially the twenty-one fleas of November 11th, 1911, in Table A, of which eighteen did not feed. This was due to a sudden cold snap, the first breath, so to speak, of winter.

two or three days previously to being put on), it is quite easy to distinguish those which have been fed from those which have not availed themselves of the opportunity of doing so. In this way useful controls are obtained for determining whether the fleas contained any flagellate infection before being used for putting on the infected rat.

TABLE A.—Fleas Examined within Twenty-four Hours after being put on an Infected Rat, to show the Numbers that had or had not Fed, and the Numbers of those that had Fed but in which no Flagellates were Found.

Date on which the fleas were examined.	Time since fleas put on infected rat.	Number of fleas put on the rat.	Number of fleas apparently not fed and containing no flagellates.	Number of fleas which appeared to have fed and which contained: (a) Stages of T. lewisi.	(b) No flagellates.
5 : vii : '10	6 hours	3	1	2	0
19 : vi : '11	12 ,,	10	2	8	0
13 : ii : '13	15 ,,	14	3	11	0
23 : v : '11	18 ,,	4	1	3	0
26 : v : '11	18 ,,	12	1	7	4
25 : i : '13	18 ,,	9	3	5	1
30 : i : '13	18 ,,	5	1	4	0
6 : ii : '13	18 ,,	8	3	5	0
4 : iii : '13	20 ,,	14	12	2	0
10 : vi : 10	24 ,,	4	2	2	0
6 : vii : '10	24 ,,	3	2	1	0
17 : iii : '11	24 ,,	5	1	3	1
15 : vi : '11	24 ,,	11	6	4	1
26 : ix : '11	24 ,,	17	7	8	2
30 : ix : '11	24 ,,	22	2	18	2
3 : x : '11	24 ,,	13	1	12	0
6 : x : '11	24 ,,	14	2	11	1
7 : xi : '11	24 ,,	17	2	11	4
11 : xi : '11	24 ,,	21	18	2	1
17 : xi : 11	24 ,,	17	5	10	2
24 : xi : '11	24 ,,	20	2	12	6
5 : xii : '11	24 ,,	12	3	7	2
12 : xii : '11	36 ,,	6	1	5	0
2 : vii : '12	24 ,,	14	8	6	0
20 : vi : '11	24 ,,	14	3	8	3
Total . . .		289	92	167	30
Percentage . .		100	31·83	57·79	10·38

From Table A it is seen that of 289 fleas which were put on infected rats, 92 (31·83 per cent.) had not fed and contained no flagellates, 167 (57·79 per cent.) had fed and contained T. lewisi, and 30 (10·38 per cent.) had fed but contained no flagellates.

In addition to these negative data we have had the opportunity of comparing our stock of fleas with another stock which was actually infected with Leptomonas pattoni. When one of us (E.A.M.) was in Paris in January, 1913, he was very kindly presented by Dr. E. Chatton, of the Pasteur Institute, with some living fleas (Ceratophyllus fasciatus) from a stock infected with Leptomonas pattoni. These fleas were brought back to London and a fresh breeding-cage colonized with them. The fleas were left to breed for a year, during which time the rat in the breeding-cage was changed frequently, but none of the rats put in acquired any trypanosome-infection. When the fleas were examined at the beginning of 1914, they had multiplied enormously, and were found to contain Leptomonas-infections. We did not keep any exact records of our dissections of the Leptomonas-fleas, but, roughly speaking, about 50 per cent. of the fleas contained teeming infections. The leptomonads appear to establish themselves in the fleas as readily as does T. lewisi, perhaps more so, since, as will be seen from Table B (1), barely more than 14 per cent. of our stock of fleas contained swarming infections when exposed permanently to infection with T. lewisi in the infected breeding-cage, and Table B (2), if we count only those known to have fed on an infected rat, not less than six, not more than fourteen days previously, gives but a slightly higher percentage (21·19).

We may conclude, therefore, from a comparison of our stock of fleas with those bred from Dr. Chatton's stock infected with the leptomonad, that, had our stock also been infected with leptomonads, we should not have failed to find fleas containing leptomonads in those fed on clean rats in the first place, and secondly, that the percentage of fleas

TABLE B.—Summary of the Condition of Fleas known to have Fed on Infected Rats. (1) Fleas taken at Random from the Infected Breeding Cage.

Date. (Fleas dissected.)	Trypanosomes.				Experimental infectivity of the flea.
	Fleas.	None.	Scanty.	Swarming.	
18 : ii : '10	3	1	1	1 (r)	Infection produced by 5 fleas of which 3 were dissected (Table J).
23 : iii : '10	4	1	2	1 (s)	
30 : iii : '10	6	1	3	2 (s)	
5 : iv : '10	6	0	3	3 (1r, 2s)	
8 : iv : '10	2	0	1 (si)	1 (si)	
10 : iv : '10	2	1	1	0	
22 : iv : '10	4	3	0	1 (s)	
3 : v : '10	4	4	0	0	
25 : vii : '10	5	2	3	0	
27 : vii : '10	3	1	2	0	
5 : viii : '10	5	2	3	0	One positive (Table K).
6 : ix : '10	3	3	0	0	
7 : ix : '10	4	3	1	0	
14 : ix : '10	3	0	2	1 (s)	
15 : ix : '10	3	0	3	0	
22 : ix : '10	3	1	1	1 (s)	One positive (Table K).
23 : ix : '10	1	1	0	0	Negative (Table K).
24 : ix : '10	1	1	0	0	
26 : ix : '10	2	1	0	1 (r)	
27 : ix : '10	3	3	0	0	
28 : ix : '10	3	3	0	0	
29 : ix : '10	3	2	0	1 (ir)	
3 : x : '10	2	2	0	0	
6 : x : '10	2	0	1	1 (r)	
10 : x : '10	4	1	2	1 (r)	
13 : x : '10	3	3	0	0	All negative (Table K).
14 : x : '10	3*	3	0	0	One positive (Table K).
17 : x : '10	3	1	1	1 (r)	All negative (Table K).
18 : x : '10	3	2	1	0	,, (Table K).
24 : x : '10	3	3	0	0	,, (Table K).
25 : x : '10	3*	3	0	0	One positive (Table K).
4 : xi : '10	3	2	1	0	,, (Table K).
8 : xi : '10	3	2	1	0	All negative (Table K).
15 : xi : '10	3	2	0	1 (r)	One positive (Table K).
16 : xi : '10	3	1	1	1 (r)	All negative (Table K).
21 : xi : '10	2	2	0	0	Both negative (Table K).
22 : xi : '10	2	2	0	0	,, (Table K).

Date. (Fleas dissected.)	Trypanosomes.				Experimental infectivity of the flea.
	Fleas.	None.	Scanty.	Swarming.	
29 : xi : '10	1	1	0	0	
30 : xi : '10	2	1	0	1 (r)	
1 : xii : '10	2	0	1	1 (s)	
5 : xii : '10	5	1	4	0	Injection of sal. gl. negative.
6 : xii : '10	2	0	2	0	
7 : xii : '10	2	0	2	0	
9 : xii : '10	2	2	0	0	Both negative (Table K).
13 : xii : '10	3	2	1	0	All negative (Table K).
14 : xii : '10	3	2	1	0	,,
15 : xii : '10	6	5	1 (sr)	0	Fleas infected rat 239, stomachs rat 257 (Table I).
12 : i : '11	5*	5	0	0	Fleas positive (Table I).
13 : i : '11	6	4	2 (r)	0	Stomachs injected, positive (Table I).
26 : i : '11	6	5	1 (ir)	0	Negative (Table I).
26 : i : '11	6*	6	0	0	Fleas infected rat 251, stomachs rat 261 (Table I).
27 : i : '11	5*	5	0	0	Fleas positive (Table I).
14 : ii : '11	10	2	6	2	Stomachs injected, 2 positive.
28 : ii : '11	10	7	2	1 (r)	Stomachs positive, recta negative.
7 : iii : '13	8	8	0	0	
13 : iii : '13	4	0	1 (sr)	3 (2s, 1sr)	
14 : iii : '13	3	0	0	3 (1s, 2sr)	
17 : iii : '13	8	8	0	0	
28 : iii : '13	9	5	4 (3s, 1sr)	0	
31 : iii : '13	7	7	0	0	
4 : iv : '13	5	4	0	1 (sr)	
7 : iv : '13	6	0	3 (sr)	3 (s)	
10 : iv : '13	6	1	4 (1s, 3sr)	1 (sr)	
14 : iv : '13	2	0	1 (s)	1 (i)	
Total .	249	144	70	35	
Percentage	100	57·83	28·11	14·06	

The batches marked * were fleas of which the stomachs and other organs were kept for injection into rats, and were therefore examined hastily and imperfectly.

s = stomach ; r = rectum ; i = intestine.

TABLE B.—Summary of the Condition of Fleas known to have fed on Infected Rats. (2) Fleas fed at Definite Periods.

Age of infection in flea (approximately).	Date.	No. of fleas.	Fleas containing Trypanosomes.		
			None.	Scanty.	Swarming.
6 hours	14 : vi : '10	4	0	1 (s, r)	3 (2s, 1sr)
,, ,,	5 : vii : '10	2	0	1 (s)	1 (s)
12 ,,	28 : iii : '11	5	0	2 (s)	3 (1s, 2sr)
,, ,,	19 : vi : '11	9	1	2 (1s, 1r)	6 (5sr, 1r)
18 ,,	21 : iii : '11	9	0	6 (s)	3 (1s, 2sr)
,, ,,	23 : v : '11	4	1	1 (s)	2 (sr)
,, ,,	26 : v : '11	11	4	3 (2sr, 1r)	4 (1s, 3sr)
,, ,,	25 : i : '13	6	1	2 (1sr, 1r)	3 (2s, 1sr)
,, ,,	30 : i : '13	4	0	2 (1s, 1sr)	2 (sr)
,, ,,	6 : ii : '13	5	0	1 (si)	4 (2s, 2sr)
21 ,,	4 : iii : '13	2	0	0	2 (s)
24 ,,	10 : vi : '10	4	2	2 (1sr, 1r)	0
,, ,,	6 : vii : '10	3	2	1 (r)	0
,, ,,	17 : iii : '11	5	2	1 (s)	2 (sr)
,, ,,	15 : vi : '11	5	1	4 (s)	0
,, ,,	27 : vii : '11	28*	10?	12	6
,, ,,	1 : viii : '11	11*	8?	1	2
,, ,,	3 : viii : '11	29*	12?	13	4
,, ,,	30 : ix : '11	10	2	6 (4s, 2sr)	2 (1s, 1sr)
,, ,,	3 : x : '11	20	2	10 (6s, 4sr)	8 (6s, 1sr, 1r)
,, ,,	7 : xi : '11	13	1	4 (1s, 1sr, 2r)	8 (4s, 4 sr)
,, ,,	11 : xi : '11	12	1	9 (7s, 2sr)	2 (2s)
,, ,,	17 : xi : '11	13	2	8 (5s, 2sr, 1r)	3 (2s, 1r)
,, ,,	24 : xi : '11	3	1	1 (s)	1 (s)
,, ,,	5 : xii : '11	12	2	6 (4s, 2sr)	4 (3s, 1sr)
,, ,,	9 : xii : '11	18	6	12 (s)	0
,, ,,	25 : vi : '12	10	0	4 (2s, 2sr)	6 (1s, 5sr)
,, ,,	2 : vii : '12	9	2	2 (1s, 1sr)	5 (1s, 4sr)
,, ,,	24 : vi : '13	12	0	7 (2s, 3sr, 2r)	5 (sr)
36 ,,	20 : vi : '11	6	1	0	5 (4s, 1sr)
48 ,,	11 : vi : '10	6	5	1 (r)	0
,, ,,	16 : vi : '10	5	5	0	0
,, ,,	7 : vii : '10	5	5	0	0
,, ,,	18 : iii : '11	3	2	0	1 (s)
,, ,,	26 : iv : ' 11	6	2	4 (1s, 2sr, 1r)	0
,, ,,	26 : vi : '12	10	1	3 (sr)	6 (5sr, 1s)
,, ,,	3 : vii : '12	10	4	3 (r)	3 (1s, 2r)
60 ,,	30 : iii : '11	4	3	1 (sr)	0
,, ,,	11 : vii : '12	16	2	12 (1s, 3sr, 8r)	2 (1s, 1sr)
3 days	7 : ix : '09	4	2	2 (1s, 1r)	0
,, ,,	16 : ix : '09	5	4	1 (s)	0
,, ,,	23 : ix : '09	3	2	0	1 (s)

Age of infection in flea (approximately).	Date.	No. of fleas.	Fleas containing Trypanosomes.		
			None.	Scanty.	Swarming.
3 days	27 : xi : '09	5	1	2 (sr)	2 (1s, 1r)
,, ,,	17 : vi : '10	5	5	0	0
,, ,,	8 : vii : '10	4	4	0	0
,, ,,	27 : iv : '11	10	6	4 (2s, 1sr, 1r)	0
,, ,,	27 : vi : '12	10	1	5 (1s, 2sr, 2r)	4 (1s, 3sr)
,, ,,	4 : vii : '12	12	6	5 (1s, 1sr, 3r)	1 (r)
$3\frac{1}{2}$,,	12 : vii : '12	16	4	8 (1s, 7r)	4 (1s, 3r)
4 ,,	8 : x : '09	6	1	4 (sr)	1 (sr)
,, ,,	15 : x : '09	8	4	2 (sr)	2 (sr)
,, ,,	19 : ix : '10	3	0	1 (sr)	2 (1s, 1sr)
,, ,,	28 : iv : '11	10	5	5 (1s, 4sr)	0
,, ,,	5 : vii : '12	12	9	3 (sr)	0
,, ,,	18 : vii : '12	12	8	3 (r)	1 (r)
5 ,,	9 : x : '09	2	2	0	0
,, ,,	22 : vi : '10	2	0	0	2 (sr)
,, ,,	20 : ix : '10	3	2	1 (i)	0
,, ,,	29 : iv : '11	10	6	1 (s)	3 (r)
6 ,,	20 : vii : '12	12	7	5 (r)	0
7 ,,	1 : v : '11	4	3	1 (s)	0
$7\frac{1}{2}$,,	26 : v : '13	6	2	2 (r)	2 (1sr, 1r)
8 ,,	12 : x : '09	2	2	0	0
,, ,,	19 : x : '09	5	3	1 (r)	1 (r)
,, ,,	17 : iii : '13	4	0	1 (sr)	3 (1sr, 2r)
,, ,,	5 : v : '13	10	3	2 (1s, 1r)	5 (2sr, 3r)
,, ,,	19 : v : '13	6	5	1 (sr)	0
,, ,,	2 : vi : '13	6	3	0	3 (r)
,, ,,	9 : vi : '13	7	1	3 (1sr, 2r)	3 (sr)
$8\frac{1}{2}$,,	27 : v : '13	6	2	2 (1sr, 1r)	2 (sr)
9 ,,	11 : iii : '13	6	3	1 (s)	2 (sr)
,, ,,	18 : iii : '13	3	1	1 (sr)	1 (s)
,, ,,	13 : v : '13	6	6	0	0
,, ,,	20 : v : '13	6	6	0	0
,, ,,	3 : vi : '13	6	2	3 (1s, 2r)	1 (sr)
10 ,,	12 : iii : '13	4	2	2 (r)	0
,, ,,	19 : iii : '13	6	3	2 (1s, 1r)	1 (r)
,, ,,	14 : v : '13	8	7	0	1 (r)
11 ,,	13 : iii : '13	2	1	1 (r)	0
12 ,,	29 : vi : '10	1	1	0	0
14 ,,	1 : vii : '10	2	2	0	0
Grand total . . .		609	230	223	156
Percentage . . .		100	37·77	36·62	25·61
Total of six days and over .		118	65	28	25
Percentage . . .		100	55·08	23·73	21·19

* Stomachs only examined.

containing flagellates would have been far higher than is shown by our tables, in fleas exposed to infection by T. lewisi.[1]

(c) Notes on the Histological Structure of the Stomach of the Flea.

We shall have occasion, when describing the developmental cycle of the trypanosome in Part II below, to relate how the trypanosome penetrates into the epithelial cells of the stomach of the flea and goes through a process of multiplication within them. It is a necessary preliminary, therefore, to understanding the effects of the parasites that we should preface our description of their development by some remarks upon the structure and contents of the flea's stomach; and in the following section we give an account of our observations upon these matters, without claiming to have added anything to the scientific knowledge of insect histology.

The histology of the digestive tract of insects has been the subject of

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[1] Nöller (1912), discussing the question of the leptomonas-infection of the fleas, remarks, p. 398, that since the larva of the flea acquires the infection, adult fleas bred in a cage can be infected, and that consequently "the arrangement of the experiments ('Versuchsanordnung') of Minchin and Thomson, who used fleas bred in a rat-cage, does not correspond to the requirements ('Anforderungen')." We are at a loss to understand to what this criticism applies or what are the "Anforderungen" to which Nöller refers. At the time Nöller wrote we had published only our three preliminary reports. The first two of these (1910, 1911, 1) refer only to the transmission of T. lewisi by fleas, and it is sufficiently obvious that the presence of leptomonads in the fleas could not affect in any way the value or significance of positive results obtained in experiments on the transmission of the trypanosomes, since, ex hypothesi, the trypanosome and the leptomonad parasite are in no way connected. Our third report (1911, 2) gave an account of the intracellular multiplication of T. lewisi in the flea's stomach, a discovery which Nöller himself has confirmed, and which also would be quite unaffected by the presence of leptomonads in the fleas. Nöller's criticism appears to us, therefore, both premature and superfluous; premature, because our stock of fleas was not, as a matter of fact, infected with leptomonads; and superfluous, because, even if the fleas had been infected with leptomonads, it would have made no difference to the experiments and observations which Nöller criticises.

numerous memoirs, and its general characteristics are very well known. It would be beyond the scope of this memoir to attempt to discuss this subject in detail or to cite the very copious literature dealing with it; but of recent works we may refer more especially to the very excellent monograph of Léger and Duboscq (1902), who have studied the intestinal epithelium of Tracheata from the same point of view as ourselves, that is to say, with the object of describing the changes produced in the epithelium by parasites (gregarines) attacking the cells, None of the insects studied by Léger and Duboscq, however, were of blood-sucking habit and the stomach-epithelium of the flea differs in a number of points from any of the epithelia described by the French authors.

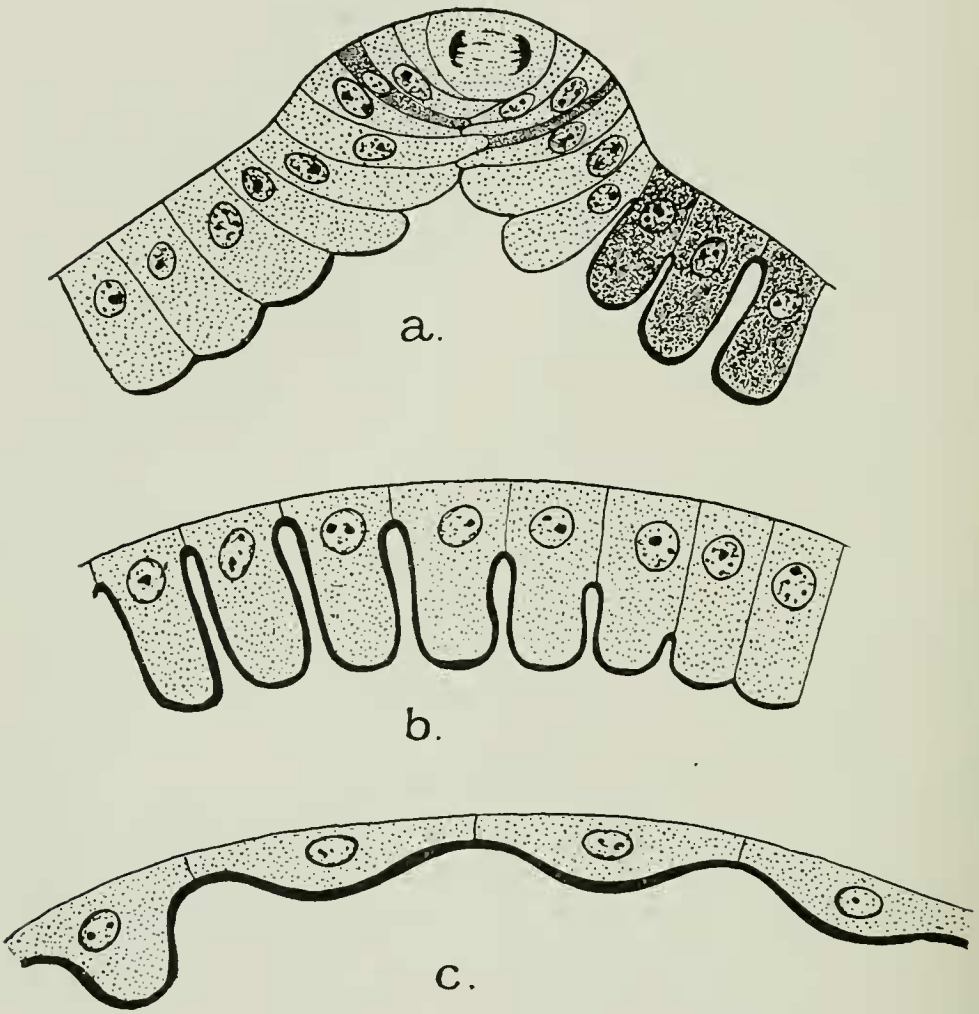
The wall of the stomach consists of the following principal layers, counted from within outwards (Pl. 39, fig. 126): (1) the lining epithelium, (2) a layer of circular muscle fibres, and (3) a layer of longitudinal muscle fibres. In addition to these layers, which are very easily seen, there are to be found also, though by no means in every section, flattened epithelial cells external to, and in contact with, the lining epithelium, between it and the circular muscle-layer; they occur sparingly and far apart, but appear to represent an integral and perhaps primitive constituent of the wall of the mid-gut. Similar flattened cells are found here and there on the Malpighian tubules, the wall of which is similar in histological composition to the stomach-wall if the latter be imagined as reduced to the lining epithelium and the flattened cells alone, without the muscle-layers.

We are concerned here only with the lining epithelium of the stomach, but it may be mentioned in passing that the circular muscle-fibres occur as bands or separate rings with considerable intervals between them, and consequently do not appear in every transverse section of the stomach. The longitudinal muscles are also separated from one another by intervals, a fact at once apparent in the transverse section, in which the muscles are seen cut across and in which it can further be seen in a well-preserved section, that each longitudinal muscle-fibre is connected to its neighbours by a delicate membrane, appearing as a fine line running between each adjacent pair of muscle-bands and forming a delicate sheath or investment round the whole stomach. The circular muscle-layer is continued on into the intestine, where it forms a continuous investment without intervals between the bands; the longitudinal muscles end at the pylorus posteriorly and start anteriorly behind the proventriculus, which has its own system of musculature, running for the most part in oblique bands arranged symmetrically right and left. Each muscle-band in the stomach-wall is a single, transversely-striated fibre, in which an occasional elongated nucleus is seen, embedded in a small quantity of protoplasm.

The following account of the epithelium and contents of the stomach

applies, unless otherwise stated, to sections of the stomach fixed with Flemming's fluid and stained with iron-hæmatoxylin followed by

TEXT-FIG. 2.



Diagrammatic representations of sections of the epithelium of the flea's stomach, to show the various conditions: *a*, to show a section through an epithelial crypt from which clear, regenerated epithelium is arising on all sides, while on one side three black, degenerated cells are seen (compare fig. 316, Pl. 44); *b*, to show the manner in which the border of the cells arises in relation to the gradually-developed separation of the cells from one another; *c*, to show the transition from the ordinary, columnar type of epithelium to the flattened type.

Lichtgrün-picric (see the following section dealing with technique). If such a section through a number of stomachs—all taken from a batch

of fleas dissected at the same sitting and at the same interval of time after having fed on the infected rat, all preserved in the same way, all stuck on the same slice of liver, cut and stained simultaneously—be examined even in the most cursory manner, very considerable differences are seen between the stomachs in one and the same microtome-section. These differences affect both the epithelium and the contents. The epithelium varies, in the first place, in the form of the cells, from flattened to columnar, and secondly, in the staining reactions of the cells. The contents of the stomach, that is to say the blood-débris, vary greatly in colour, staining in some cases deep opaque black, or less deeply in various shades of grey, in other cases, however, bright yellow.

The variations in the form of the epithelial cells are to be ascribed to the differences in the degree to which the stomach is dilated by the ingested blood. In a gorged flea the distension of the stomach stretches the epithelium until the cells become thin and flattened; but when the flea is hungry, or has taken in a small quantity of blood, or when the quantity ingested has become reduced by digestion and absorption, the epithelium resumes what may be considered its normal columnar form. Every gradation between the flattened and columnar conditions can be found in different sections or in different parts of one and the same section.

The variations in the staining reactions of the epithelial cells depend, in the first place, on the age or senescence of the cells. It is a matter of common knowledge that the lining epithelium of the mid-gut of insects is continually being thrown off and regenerated. The ordinary epithelial cells do not multiply and no mitoses are ever found in them; the centres of regeneration are the so-called epithelial crypts, each representing morphologically a small diverticulum of the epithelium in which the approximation of the cells usually obliterates the cavity and produces a solid, bud-like mass of cells (Text-fig. 2, *a* and Pl. 44, figs. 314, 316). When a flea's stomach, containing a certain amount of ingested blood, is plunged into a fixative, the epithelial crypts are very easily seen with a hand-lens or with the naked eye as little opaque white spots in the semi-transparent stomach-wall, very conspicuous against the reddish-brown background of the stomach-contents. In the sections it is common to find mitoses in such a crypt, especially towards its fundus (Text-fig. 2, *a*). As the cells multiply they are pushed upwards to the general level of the epithelium and outwards from the crypt to replace the old epithelial cells which, having degenerated, are cast off from the wall into the lumen of the stomach, and are digested there.

The young, freshly-regenerated epithelial cells have the cytoplasm clear, staining light-grey, and are relatively poor in granulations; the older cells, on the contrary, have the cytoplasm full of granules that stain very deeply, until finally the whole cell, including its nucleus

becomes black and opaque. Consequently, the epithelia of different stomachs show very varied appearances. A recently regenerated stomach will show clear epithelium all round, and, according to the time that has elapsed since regeneration, there may be no detached cells in the lumen of the stomach, or there may be a certain number of detached black cells, or there may be still, here and there, isolated black cells or patches of such cells in situ in the epithelium or in process of being cast off from it. On the other hand, a stomach which is about to be regenerated shows very dark epithelium all round, and in places this may be in process of rejection and replacement from the crypts, in which the cells have clear cytoplasm. The condition of the epithelium may vary in different parts of the same stomach, and from what we have observed we have gained the impression that the regeneration proceeds usually from before backwards, so that the anterior part of the stomach is further advanced in degeneration or regeneration, as the case may be, than the posterior region. We find in our preparations all possible conditions of the epithelium in different stomachs in one and the same microtome-section, and we have not been able to establish any definite relation between the feeds of the flea and the regeneration of the epithelium, but we have not paid sufficient attention to this point to be able to state positively that no such relation exists; the differences seen in the epithelia of flea-stomachs examined at the same interval of time after feeding may be due to inequalities in the rate at which digestion proceeds.

In addition to what appears to be the normal process of senile decay, in which the cells take up the iron-hæmatoxylin stain very deeply and become black and opaque, we have observed a second mode of degeneration, which we are inclined to ascribe to the action of the trypanosomes, since in all cases where it occurs in our preparations there are trypanosomes to be found in the stomach, and frequently in the degenerated cells themselves. In this second type of degeneration the black-staining granules in the cell diminish in quantity, without, however, disappearing entirely, while the cytoplasm of the cell stains yellow (Pl. 39, fig. 133; Pl. 40, fig. 140); hence we have generally referred to this condition in our notes as "yellow necrosis." In all the stomachs in which we have found it the blood-débris is also stained yellow, and it is often very difficult to make out the precise boundary of the necrosed cell-body, or to distinguish the cells from the débris when they lie free in it (Pl. 39, fig. 125), except by the presence of the nucleus and of a certain number of black granules in the cytoplasm of the necrosed cell. Indeed, our first impression was that the yellow colouring matter of the blood had in some way penetrated into the cell and stained its cytoplasm, but there can be no doubt that this idea is an illusion and that the yellow colour, both of the blood-débris and of the necrosed cells, is due to the picric

acid in the Lichtgrün-picric staining combination, though it is, of course, possible that the substance, whatever it may be, which stains yellow in the blood-débris may have become infiltrated into the dead cells and given them their peculiar staining properties.

The variations in the staining reactions of the contents of the stomach are more difficult to explain. There appear to be two types of staining after the use of the iron-hæmatoxylin-Lichtgrün-picric combination, one in which the contents stain in various shades of grey up to black, with a greenish tinge, and one in which they stain a bright lemon-yellow. It is not possible to bring these two types of staining into one series, as there is no transition between them; the grey-black and the yellow types occur side by side in different stomachs in one and the same microtome-section, and each stomach shows either the one or the other condition through the whole series of sections. So far as our observations permit us to generalise, the grey-black series represent the normal stages of the digestion of the blood; the yellow reaction appears to be due to some abnormal condition.

The blood ingested by the flea is very soon affected by the digestive action of the stomach, and the red corpuscles cease to be recognisable within a few hours after digestion. In the middle period of digestion, that is twenty-four hours, or thereabouts, after feeding, the blood has become thick, viscous and brick-red in colour, and contains immense numbers of irregularly shaped grains of all sizes, but for the most part coarse and large. Towards the end of digestion, forty-eight hours or so after feeding, the stomach contents are fluid and watery, dark brownish-black in colour, and the grains are much diminished in number and in size.

In the sections, taking first the grey-black series, the blood in the earlier phases of digestion (eighteen to twenty-four hours) usually consists of densely-packed grains and spherules, varying in size from very coarse to very fine, and staining intensely black. Between the grains there is visible a coagulated albuminous matrix, stained greenish with the Lichtgrün-picric combination. The stomach-contents fill the whole section and adhere closely to the epithelial cells, penetrating down between them when they have the columnar form, but in the centre of the section there is generally a clear patch, circular in outline, which is seen to owe its clear appearance to the fact that coarse grains are absent, and it consists only of the albuminous matrix with finer granules. Hence the digestion, or more probably the passage backwards towards the rectum of the indigestible remnants, of the blood-débris appears to proceed from the centre of the section—that is to say, from the axial region of the stomach—towards the periphery.

As the digestion proceeds, the grains in the débris become smaller

and stain less deeply; consequently the stomach-contents stain grey, in varying shades of darkness, while the matrix still shows the greenish hue. In stomachs thirty-six hours after feeding the contents of the stomach are generally greatly diminished in quantity, and are absorbed in the centre of the lumen, leaving a clear space of variable form, while round the periphery the greenish-grey débris adheres close to the epithelium. Leucocytes, especially the polymorphonuclear forms, can be recognised in the blood twenty-four hours after feeding, but at thirty-six hours we have not found them. Owing to the lighter tints of the stomach-contents at thirty-six hours, the trypanosomes free in the blood-débris can be seen more easily, in contrast with the earlier state of affairs.

In the yellow stomachs the contents appear at first almost uniform, but on close examination they are seen to consist of closely-packed granular substance, all of which, both granules and matrix, is coloured by the picric acid in the staining combination used. The first point that strikes one immediately is that the contents in such stomachs are large in quantity and fill the whole stomach, or show but a slight amount of absorption towards the centre of the section, even at thirty-six hours, when the contents of the grey-black stomachs are considerably diminished. The epithelium of the yellow stomachs may vary from the flattened to the columnar form, but the normal cells stain grey or black, in sharp contrast with the yellow contents.

It seems obvious from these data that the yellow stomachs represent an abnormal condition; we have endeavoured, not very successfully, to find a relation between this condition and either the presence of trypanosomes, on the one hand, or the state of the epithelium on the other.

In the yellow-staining stomachs which we have studied we have found trypanosomes to be present in the stomach in every case except one, and in that case there were attached clumps of crithidial forms immediately behind the pylorus, showing that the stomach-phase was over. But on the other hand, we have found the grey-black condition of the contents in well-infected stomachs also, showing at least that, if the yellow condition is in any way due to the parasites, they do not always produce that effect. On the other hand, in those cases in which we have found no trypanosomes at all, either in the stomach or outside it, the contents are always in the grey-black condition. A significant circumstance is, perhaps, the fact that we have only found the "yellow necrosis" of the cells in stomachs with yellow-stained contents.

As regards the condition of the epithelium, we have found the yellow condition of the contents associated (1) with epithelium black all round and in process of being cast off, or (2) with epithelium mostly clear, but with black patches of cells in situ or detached; in one such stomach the

first condition is found in the anterior half, the second in the posterior. We can state, therefore, that in our experience the yellow-stained contents occur only in stomachs about to be regenerated, or in process of regeneration, or very recently regenerated. But, again, we have found the grey-black condition in stomachs that appeared also to have undergone regeneration very recently, which makes it difficult to correlate this condition with the process of regeneration. The question of the significance and cause of the yellow-staining condition of the stomach-contents must be left an open question at present; the data to hand do not suffice for drawing decisive conclusions, and it would lead us too far to attempt further investigations upon this problem. On the whole, however, it seems at least probable that we are dealing with an abnormal state of the digestive processes towards which the trypanosomes are a contributory cause, if not the sole one.

As already stated, the different conditions of the stomach-contents described above are those seen after staining with iron-hæmatoxylin and Lichtgrün-picric. After the use of Giemsa's stain the colour of the contents differs considerably in different cases.

Most of our sections stained with Giemsa were fixed with Maier. In those in which the trypanosomes were best stained and show the flagella clearly and sharply the grains and spherules of the débris are coloured for the most part orange-pink, especially in those stomachs in which the digestion of the blood is further advanced (figs. 109, 113, Pl. 38); in the earlier stages of digestion many of the larger grains and masses in the débris are stained deep purple, making the contents of the stomach more opaque. In one of our series preserved in Flemming, consisting in all of seven slides, the first six were stained with the iron-hæmatoxylin-Lichtgrün-picric combination, the seventh with Giemsa's stain; on this seventh slide there are sections of four stomachs, two of which, on the other six slides, show grey-black contents, while the remaining two have the contents yellow in colour. In the Giemsa-stained slide the blood-débris shows a coloration very different after Flemming to that which it shows after Maier, being stained a bluish-green tint. The stomachs of the yellow type are slightly more blue in tint than those of the grey-black series, but otherwise the difference between them is but slightly marked.

Having now described the chief variations in the conditions of the stomachs and their contents, or at least those differences which are obvious upon the most cursory inspection of the sections, it remains to give a more detailed account of the epithelial cell. In any given stomach the cells show great individual variation in form and structure, but, nevertheless, it is not possible to divide them into distinct classes. There are no special glandular or secreting cells, as described by Léger and Duboscq in other insects, and all the cells of the general epithelium

of the stomach of the flea are to be regarded as equipotential, the differences visible between them being merely the expression of varying physiological conditions in relation to their changing environment, on the one hand, or to their constitutional vigour or senescence, on the other. Hence it is possible to give a generalised description of the cells, beginning first with the normal, healthy cell and dealing afterwards with the changes it undergoes in the process of degeneration.

The epithelial cells are produced, as already stated, in the "crypts of regeneration," which have been described in various insects by Léger and Duboscq. In the flea these structures appear usually as solid, bud-like cell-masses that often project outwards from the wall of the stomach to a considerable extent (Pl. 44, figs. 314, 316) beyond the level of the muscle-layers, which pass on either side of them. Internally the crypts do not rise up beyond the general level of the epithelium. The closely-packed cells of the crypts show distinct limits, and do not form a syncytial mass of protoplasm, as described by Léger and Duboscq (l. c., p. 410) in the larva of Anthrenus verbasci, for example. At the fundus of the crypt mitoses are often found, sometimes in two cells simultaneously in the same crypt; in other cases all the nuclei are in the resting state. Doubtless the crypts have periods of active multiplication, alternating with periods of repose, as in other insects. The crypts are often seen to be marked off from the general epithelium by slender dark cells, the "cellules de recouvrement" described by Léger and Duboscq (l. c., Pl. II, fig. 2, *c. r.*; p. 388). The crypts appear to have the monopoly of cell-production in the stomach of the flea. We have not found basal cells, "cellules de remplacement," in the general epithelium.

By multiplication and increase in their numbers the cells are pushed outwards on all sides from the crypt to take their place in the general epithelium (Pl. 44, fig. 316, and Text-fig. 2*a*). The young epithelial cells seen in the immediate neighbourhood of the crypts are columnar cells, roughly rectangular in form, and generally about twice as high as they are broad. The lateral boundaries of the cell are approximately parallel, and each cell is in contact with its adjacent neighbours for its whole length. The free, apical surface of the cell is convex, and on this side is developed a very distinct, thick border, at first covering only the upper surface of the cell, which projects like a dome towards the lumen of the stomach.

The further development in the form of the cell consists in an extension of the upper free surface, brought about by the cells becoming free and separated from one another at their sides, first at their apices and then downwards along almost the whole length of the side of the cell, till finally each cell is connected with the adjacent cells only by a narrow isthmus at its base (Text-fig. 2*b*). As the cell

becomes free the border develops also on the exposed surface, so that, instead of being confined to the apex of the cell, it extends down the vertical sides also (Pl. 40, fig. 136). This process of separation between the cells has an obvious significance in connection with the process of flattening which they undergo when the stomach is dilated after feeding; it can be regarded as an adaptation to the blood-sucking habit. When the flea gorges itself each cell is so stretched that its tallest part in the vertical direction is scarcely thicker than the nucleus, which bulges out the middle part of the cell in an even curve towards the lumen of the stomach, while towards the periphery the verticle height of the cell diminishes to the isthmus connecting it with its neighbours (Text-fig. 2 *c*). As the cell resumes the columnar form the nucleus remains at or near the base, as a rule, and the cytoplasm of the cell is heaped up over it. In the extreme columnar form the apex of the cell is generally slightly expanded, the middle region more narrowed, so that spaces are left between adjacent cells, into which a considerable quantity of blood-débris penetrates (Pl. 39, fig. 126). The nucleus is usually situated at the base of the cell, but occasionally towards the apex (Pl. 40, fig. 136). The border clothes the whole free surface of the cell, whether flattened or columnar, and is of considerable thickness over the apex and the sides, becoming thinner as it approaches the isthmus, but in the columnar form of the cell, when its apical region is expanded, the border may be thinner, as if stretched, at the apex of the cell (Pl. 40, fig. 144).

The blood-débris has a great tendency to adhere closely to the border, so much so that the border is often more sharply marked off from the cell-contents within than from the blood-débris without, in the sections, but places can be found occasionally where the blood-débris has split away from the epithelium, leaving the border distinct and sharp. The border appears usually homogeneous and refringent, though in some preparations indications are seen of a vertical striation, as if it were composed of little darkly-stained rods, placed at right angles to its two limiting surfaces, and separated by intervening substance of lighter colour (Pl. 39, fig. 129, and Pl. 40, fig. 142). After sublimate-fixation the border is colourless, but when stained with iron-hæmatoxylin the blood-débris adhering to it often hinders the extraction of the stain and at these spots it remains black; when the hæmatoxylin is extracted it tends to take up the green from the Lichtgrün-picric mixture (Pl. 40, fig. 147). With Giemsa after sublimate-fixation it stains a pinkish-yellow. After Flemming-fixation the border is yellowish, as if tinged by the chromic acid in the mixture, and when this fixation is followed by the Giemsa-stain the border is coloured green (Pl. 38, figs. 99–103). There is no "bordure en brosse," or palisade of stiff rod-like cilia, external to the border, as in many insects. The condition in the flea

more resembles that figured by Léger and Duboscq for Scolopendra (l. c., pl. vi).

The border is evidently a fairly tough structure since in teased up stomachs examined fresh, the borders of cells are often seen quite empty, but retaining their shape, like shells.

The nucleus of the epithelial cell calls for no special comment: as can be seen in our figures, it is rounded or oval, with the typical structure seen in tissue-cells, namely, a distinct membrane, a reticulum containing chromatin-grains of various sizes, and one or more nucleoli which stain black, like the chromatin, after iron-hæmatoxylin. Mitoses of the usual type are found commonly in the crypts of regeneration, but we have never seen the slightest evidence of nuclear division in cells forming part of the general epithelium outside the crypts.

The cytoplasm of the epithelial cell varies at different ages. In the youngest cells bordering the crypts the cytoplasm appears more or less homogeneous and finely granular in all parts of the cell; it stains light purplish-grey or grey-black after iron-hæmatoxylin, bluish-purple after Giemsa, and no coarse granulations are to be seen. In the fully developed cell the cytoplasm has undergone local differentiation; round the nucleus, in the basal half of the cell, it has a denser texture, but above the nucleus, in the apical region, it has become of looser consistence, more spongy, so to speak, in appearance, with irregular spaces (Pl. 39, figs. 126, 127, Pl. 40, figs. 136, 144), containing fluid in the living condition, and transversed by strands of protoplasm disposed irregularly. The more the apical part of the cells is expanded the more watery its contents appear. Sometimes the apical region appears almost empty in the sections, with only a few traces of cytoplasm close under the border and at the sides. It is in this region in which the stages of the trypanosomes are most often found, and into which the parasites first penetrate.

In addition to these changes in the cytoplasm, numerous grains and enclosures of various kinds make their appearance in it. A detailed study of these granulations would require a lengthy investigation, an expenditure of time and trouble, that would go beyond the scope and objects of this work. We must confine ourselves to a brief summary of the appearances seen in our sections, without attempting to give physiological explanations of the various conditions seen. It is obvious that the bare observation that a granule is stained black by iron-hæmatoxylin or red by Giemsa's stain does not permit very far-reaching conclusions as to its nature or function in the cell; bodies of most diverse properties might agree to this extent in their reactions.

The first granulations to appear are minute grains which, whatever the fixation, Flemming or sublimate, stain black after iron-hæmatoxylin and red after Giemsa. They are seen at first chiefly at the sides of the

nucleus, between it and the cell-wall and extend up the sides of the cell close under the border. Scanty at first in the apical spongy part of the cell, they are soon deposited in this region also, appearing often in considerable numbers and varying in size from small granules to conspicuous grains, and even large masses (Pl. 40, figs. 136, 137.) The larger grains are seldom homogeneous, but appear as rings, black or red, as the case may be, with clear centre, apparently hollow (Pl. 38, fig. 99). Those of still larger size show, especially after Giemsa, darker and lighter parts disposed in various ways; inside the peripheral deeply-stained shell there may be darker grains or patches. After iron-hæmatoxylin, however, the whole mass may be opaque black, but usually shows lighter inner portions. The extent to which these granulations are developed varies in different stomachs, doubtless in relation to their secretive or absorptive activity at the moment of preservation. When a number of stomachs are cut in the same block, one stomach all through the series may show the cells clear and very free from granulations, while another stomach shows nearly every epithelial cell loaded with coarse grains in its apical region.

The red grains, as they may be termed from their distinctive reaction to Giemsa's stain, appear to be always present in greater or less quantity in the fully-developed cells of every stomach. In addition there are often found, lodged in the apical spongy region of the cell, masses of relatively large size which do not retain the iron-hæmatoxylin stain firmly throughout their substance, and consequently appear for the most part light grey in colour after this stain (Pl. 40, figs. 145, 146); after Giemsa they are either scarcely stained at all, appearing a sort of neutral tint, or they are coloured bluish-purple in various shades, sometimes very deeply, with streaks and blotches more reddish in tint (Pl. 38, fig. 102). These masses vary considerably in size and contour, and show differentiation of their substance into lighter and darker parts. With superficial examination they often simulate the intracellular stages of the trypanosomes to a remarkable degree, especially in the living condition, when they are often very conspicuous; for a long time we confused them with the spheres in the freshly teased-up stomachs, and spent much time watching them in the expectation, never of course fulfilled, of seeing them perform the characteristic movements. After we had made smears of stomachs in which these bodies were abundant, without finding any intracellular stages of the trypanosomes in such preparations, we came to the conclusion that these motionless spheres (as they appeared to be) were merely cell-products, and referred to them in our notes as "pseudospheres." Even in sections the pseudospheres often mimic the true spheres and might be confused with them at first sight, but only by an inexperienced observer who had never seen the actual intracellular stages of the trypanosome in the epithelium. The

idea occurred to us at one time that some of the pseudospheres might possibly be degenerated stages of the trypanosomes, destroyed, and in process of absorption, within the epithelial cells into which they had penetrated: but we have found no decisive evidence for this. It is most probable that the pseudospheres represent secretion-masses formed by the cell itself.

In some of the stomachs, especially in those preserved about twenty-four hours after feeding, there are to be seen dense and very conspicuous accumulations of coarse grains in the epithelial cells immediately below the border (Pl. 38, fig. 98, Pl. 40, fig. 147). The grains in question are especially distinct after fixation with Maier's fluid; they are more difficult to make out in the stomachs fixed with Flemming. The grains resemble very closely those of the blood-débris adherent to the border external to the cell, so much so that the first impression gained is that the débris has been absorbed into the cell through the border. It is easy to imagine this after iron-hæmatoxylin, which stains both these granules and the débris very black after sublimate-fixation (fig. 147); but the Giemsa-stain colours the grains within the cell differently from the débris (fig. 98), and when the digestion of the blood has gone beyond a certain point the grains inside the cell may be stained much darker with iron-hæmatoxylin than the grains in the blood-débris. It is improbable that the coarse grains of the débris would pass bodily through the border, which is to all appearances a dense, tough structure; but it is probable that these grains are formed in the cell in direct relationship with the process of absorption of nutriment from the blood.

Amongst the enclosures of the epithelial cell must be mentioned finally peculiar yellow grains which occur with great frequency in some stomachs, not at all in others. Their presence or absence is in no way connected with that of the trypanosomes, and they occur both in normal as well as in degenerating cells, though perhaps more abundantly in the latter. In the Flemming-iron-hæmatoxylin sections these grains have a brownish-yellow tint, often with a darker shell (Pl. 40, fig. 141). They vary in size from small granules up to the large grains reaching as much as 13 μ in diameter (fig. 142). Their tint also varies in depth, being usually much lighter in the larger grains. With Giemsa, after Flemming, they are stained bright green (Pl. 38, fig. 103), probably as the result of a blue stain (azure) imposed upon their original yellow tint.[1] These yellow bodies are very similar to, probaby identical

[1] A similar result is seen in the chitinous spines of the proventriculus in sections stained with Giemsa; the cuticle at the base of the spine is stained red, but that of the spine itself, from near the base to the tip, is coloured emerald green. The unstained spine is yellow in tint. Com-

in nature with the enclosures characteristic of the pericardial cells. As one of us has described elsewhere (E.A.M., 1910), the pericardial cells of the flea may be so crammed with yellowish-brown grains and spheres that the cell becomes visible with the naked eye as an opaque black spot through the integument of the living flea. In some of our stomach-sections there are also casual sections of pericardial cells which have been pulled out of the flea together with the stomach, so that we have had the opportunity of making a direct comparison between the yellow grains in the epithelial and pericardial cells. The yellow grains are probably an excretory product, eliminated by the flea under certain physiological but apparently normal conditions, and elaborated either in the epithelial cells, to be cast out into the lumen of the stomach, or in the body-cavity, to be taken up by the pericardial cells.

We come now to the process of cell-degeneration which occurs in the effete, senile epithelial cells. This process is very different in the flea's stomach from that described by Léger and Duboscq in various insects, none of them of blood-sucking habit. It is described by these authors as a "Dégénérescence mucoïde," an infiltration of the cells with mucoid substance. In the flea's stomach the process appears to be more of the nature of a fatty degeneration, combined perhaps with a mucoid infiltration.

In our sections fixed with Flemming's fluid and stained with iron-hæmatoxylin the intensely black, often perfectly opaque, degenerated cells, which are seen frequently detached completely or in process of detachment from the epithelium, are very distinct from the clear, lightly-stained cells originating from the crypts of regeneration and taking the place of the degenerated cells (Pl. 44, fig. 316). In some of our sections the stain has been over-extracted: the trypanosomes have become ghosts, faintly visible only to the practised eye, the nuclei of the epithelial cells are pale, and even the blood-débris has had its usually intense black stain reduced to a shade of brown; but the black grains and masses in the epithelial cells remain as black as ever, showing that they do not owe their colour to the stain but to the fixation, that is to say, to the osmic acid in the Flemming's fluid. Such preparations, spoilt for other purposes, are very useful for showing the gradual process of deposition of the blackened grains. First they appear as fine granules round the nucleus, near the base of the cell (Pl. 40, figs. 143, 144). Next, other, and for the most part larger masses, are deposited in the cytoplasm above the nucleus. The cell then becomes gradually filled up with black grains from below towards the apex; often an

pare also the green stain of the border, mentioned above, after Flemming and Giemsa, evidently due also to the super-position of a blue dye upon a yellow ground.

empty space is seen at the apex, immediately below the border (Pl. 40, fig. 138), but finally this, too, is filled up and the whole cell becomes an opaque black mass (fig. 139).

Very instructive is one of our series preserved in Flemming, in which there is one stomach in which nearly all the epithelium is degenerate. The sections of this stomach are spread over seven slides, six of which were stained with iron-hæmatoxylin, while the seventh, on which are sections through the hindmost region of the stomach, was stained with Giemsa. On this slide the degeneration is not so far advanced as in the more anterior region of the stomach, and in different parts even of the same section the following conditions are to be found: (1) Cells of normal type, with clear cytoplasm containing a few red granules (Pl. 38, fig. 99); (2) cells with cytoplasm of a darker bluish-purple tint, with many more red granules and amongst them a few coarser grains intensely black in colour (Pl. 38, fig. 100); (3) cells in which both the red and the black grains, but especially the latter, are greatly increased in number, leading up to (4) opaque black cells in which nothing can be focussed clearly. The black grains, it is obvious, can only owe their colour to the action of the osmic acid in the fixation, and must therefore be of a fatty nature. On the other hand there is also a marked increase of the red grains in the degenerating cells, indicating, perhaps, that in addition to deposition of fat, there is also a tendency to mucoid infiltration, as described by Léger and Duboscq. The darker tint of the cytoplasm, in so far as this is not an optical effect due to crowding of the grains, indicates that it becomes impregnated with the substances produced in the process of degeneration.

The deposition of the fat round the nucleus in the first instance indicates that the nucleus takes an active share in the process, and this is borne out by the fact that the nuclei themselves become very dark in the degenerating cells and are sometimes quite opaque.[1]

In sections of stomachs fixed with sublimate mixtures the blackening of the degenerating cells seen in the Flemming-fixed sections is conspicuously absent, so that at the first glance it is difficult to pick out the senile portions of the epithelium. More careful study of the sublimate sections shows that here the degenerated epithelium is distinguished from the regenerated by its pale, empty appearance, owing to the fat-grains having entirely disappeared, leaving empty spaces to mark their former position. This is best seen in stomachs fixed in sublimate-acetic, since, after sublimate-alcohol mixtures (Maier's and Schaudinn's) the cells are often much deformed and shrunk. In a favourable spot it is seen that the young cells, freshly produced from

[1] Léger and Duboscq have noted also that the mucoid substance is deposited first in the nucleus.

the crypts, have denser cytoplasm filling the cell throughout, except in the apical expanded portion of the cell; the cytoplasm stains deep grey or neutral tint after iron-hæmatoxylin and shows relatively few enclosures. The senile cells, on the contrary, are full of cavities, so that the cytoplasm has a spongy appearance throughout the cell and not merely in its apical region; and scattered through the spongy cytoplasm are grains, fine or but moderately coarse, which are stained black after iron-hæmatoxylin, red after Giemsa.

The difference between the senile cells after the two methods of fixation is easily explained if the grains deposited in them are principally fat. In all the sections alike the fat has been dissolved away during the process of imbedding in paraffin. In the Flemming-fixed sections, however, each fat-grain has reduced the $Os\,O_4$ to metallic osmium, and consequently is represented in the sections by a black mass, a model of the fat-globule in metallic osmium. In the sublimate-fixed sections no such reduction takes place, and the fat-globule is represented by an empty space; only the mucoid grains (if we are right in calling them so) remain in the cytoplasm, stained red or black according to the stain used.

It should be mentioned finally that after sublimate-fixations the blood-débris is stained very much blacker by iron-hæmatoxylin, and holds the stain much more tenaciously than after Flemming-fixation. This is especially true of that part of the débris which penetrates down between adjacent epithelial cells, and which often remains jet-black after all the rest of the débris has become pale in tint. In consequence the cells of the columnar epithelium in sublimate-fixed sections are often seen to be separated by black masses, which careless observation might confuse with the black stain of the degenerated cells after Flemming-fixation, especially when, as often happens in such sections, the main mass of the débris has shrunk away from the epithelium into the centre of the stomach-lumen. Such a mistake could only be made, however, with powers too low to discern that the black masses are between the cells and not in them.

The degenerated cells are thrown off bodily into the lumen of the stomach, which often contains great numbers of them in the blood-débris. There they are doubtless digested and absorbed along with the other contents of the stomach. Léger and Duboscq described a process of mucoid degeneration in which the entire cell, having a remarkable and deceptive resemblance to a gregarine, is engulphed by a basal cell; ultimately the latter also degenerates, and is thrown off with the cell it has taken in (l. c., p. 451). We have seen nothing of this sort in the flea, in which basal cells do not occur in the epithelium of the stomach.

(3) Technique.

We have already described above our methods of dissecting the flea and extracting from it the organs which it is required to examine for the presence of stages of T. lewisi. Here we propose to describe the methods by which the trypanosomes, when found, were preserved as permanent preparations for microscopic study.

The organs of the flea, extracted in the manner described above, are at once examined carefully under the microscope for the presence of trypanosomes in their various phases of development. When trypanosomes were found in any of the internal organs, after note had been taken, or sketches made, of their forms, position, and other points of interest, we proceeded to make permanent preparations of them. For this purpose the coverslip is carefully raised up, by means of the pair of fine needles that were used in the dissection of the flea, lifted off, and dropped at once with wet surface downwards into a suitable fixative. The slide is then handed to the collaborator or to an assistant, who places it bodily into a tube containing a small quantity of four per cent. solution of osmic acid. In the tube the slide remains about ten to fifteen seconds, tightly corked up, in order to fix the trypanosomes with the vapour of osmic acid. Subsequently the slide is fixed with absolute alcohol for about fifteen minutes and stained with Giemsa's stain in the usual manner.

For the fixation of the coverslip-films we used, in the earlier periods of our investigation, either Schaudinn's fluid (corrosive sublimate, saturated solution in distilled water, 100 c.c.; absolute alcohol, 50 c.c.; glacial acetic, a few drops) or sublimate-acetic ($HgCl_2$ saturated in H_2O, 95 volumes; glacial acetic, 5 volumes). Both these fixatives gave results about equally good; it is difficult to choose between them. Latterly, however, we used only Maier's modification of Schaudinn's fluid (distilled water, 200 c.c.; absolute alcohol, 100 c.c.; sodium chloride, 1·2 grm.; $HgCl_2$, 10 grm.), since this appeared to us to give better preservation, and, in particular,

less shrinkage of the bodies of the trypanosomes, than the others. The fluid being put into a large watch-glass, the coverslip is dropped into it with the film downwards. The coverslip usually sinks in the fluid and then rests on its corners on the rounded bottom of the watch-glass, so that the film itself escapes any friction or injury. The coverslips are left in the fixative from ten minutes to half-an-hour or longer (the exact time appears to be immaterial), and are then passed through 50 and 70 into 90 per cent. alcohol, where they can be kept until it is convenient to stain them.

The coverslip films were stained almost invariably with Heidenhain's iron-hæmatoxylin, using $3\frac{1}{2}$ per cent. iron-alum solution and $\frac{1}{2}$ per cent. hæmatoxylin-solution, both in distilled water. The film, after having been brought down through graded strengths of alcohol (80, 70, . . . 10 per cent.) to water was left about twenty-four hours in the iron-alum, then as long in the hæmatoxylin. Immediately before using the hæmatoxylin-solution a few drops of a saturated watery solution of lithium carbonate was added to it, drop by drop, until the solution, when shaken up, was a bright claret-red colour. After the film had been twenty-four hours in the hæmatoxylin-solution the differentiation of the stain was carried out under control by the microscope in a weak (light brown) watery solution of iron-alum. When differentiation was complete the film was washed in a current of tap-water for at least twenty minutes, then rinsed in distilled water and brought up through graded strengths of alcohol to absolute alcohol. At this stage the coverslip was usually dipped for a moment into Lichtgrün-picric solution (Lichtgrün, 1 grm.; picric acid, $\frac{1}{2}$ grm.; absolute alcohol, 100 c.c.), then washed again in absolute alcohol, passed through xylol, and mounted in pure xylol-balsam on a slide. The Lichtgrün stain must be used very rapidly, as it stains intensely.

In this way two preparations were obtained of the contents of each organ—one on the coverslip, the other on the slide—and as a rule trypanosomes were found more or less

abundantly on both of them, so that it was possible to compare corresponding phases of the development prepared by distinct methods of technique. It is very important, however, that the operation of removing the coverslip and fixing the films should be performed very rapidly and expeditiously, in order to avoid any drying taking place. The coverslip is particularly liable to dry, since the film of liquid that adheres to it is very thin; the slide, on the contrary, does not dry so quickly. A coverslip that has dried before fixation is quite useless for staining by the iron-hæmatoxylin method; the trypanosomes acquire a characteristic shiny appearance, as if they had been glazed, and when the stain is extracted in order to differentiate the preparation, it does not come out of the cytoplasm evenly, but gives a blotchy appearance, with no sharp differentiation of the nucleus or flagellum. It sometimes happens that a coverslip-film may be otherwise satisfactory, but may have dried slightly at or near the edges, thus affording opportunities for comparing the effects of desiccation on the trypanosomes with the condition of others that have never been dried. It is then seen that, in addition to the defective staining already described, the trypanosomes are flattened and distorted in various ways.

The fragments of tissue in the dissection adhere, for the most part, to the coverslip; it is not possible, however, to make out anything of trypanosomes which remain within the organs in film-preparations, and it is therefore necessary to tease up the organs well, after dissecting them out, in order to set free the trypanosomes. In the case of those phases which are attached to the gut-wall many remain so attached even when the wall is teased up, but a certain number are usually set free. When such forms are seen in the fresh film they should be dislodged, as far as possible, by tapping gently on the coverslip with a needle.

In some cases a coverslip-film which had been stained with iron-hæmatoxylin was unmounted by dissolving the Canada balsam in xylol, after the trypanosomes on it had been

studied and drawn, and the hæmatoxylin-stain completely extracted by placing it for twenty-four hours in a 3½ per cent. solution of iron-alum. The coverslip was then washed for an hour in a current of tap-water, and could then be re-stained by some other method—for example, Twort's stain. Trypanosomes that had been already drawn after the hæmatoxylin-staining could then be drawn again after being stained in a different manner. This double staining did not seem to injure the trypanosomes in any way, but it is noteworthy that after re-staining with Twort's stain they always came out a little smaller, when re-drawn with the camera lucida, than they had done previously after the hæmatoxylin-stain (compare figs. 260–63, Pl. 42, with figs. 260*a*–263*a*, Pl. 38).

When, as sometimes happened, the trypanosomes were so scanty on the coverslip as to require prolonged searching to find them, it was often very difficult to judge the right amount of extraction of the hæmatoxylin in the process of differentiation by means of iron-alum. Morever, a degree of differentiation which is sufficient for trypanosomes in the thinner parts of the film is insufficient for the thicker parts. Hence it was often necessary to unmount the preparations and differentiate them further, perhaps two or three times, before the right degree was attained. It is difficult to judge of the required differentiation by the fragments of tissue in the films, since the minute bodies of the flagellates give up the stain much more quickly than the relatively thick tissue-cells, and in a preparation in which the latter are satisfactorily differentiated the trypanosomes become mere ghosts, requiring to be re-stained altogether. The counter-stain with the Lichtgrün-picric mixture was found to show up the cytoplasm and flagellum of the trypanosomes more clearly.

However carefully the preparations have been made, it is often difficult to make out clearly and with certainty the structural details of some of the minuter phases of the life-cycle, and for this purpose the best optical apparatus was required, both as regards the objectives and the illumination

used. All trypanosomes in the permanent preparations were drawn by Miss Rhodes, under our supervision, with the camera lucida at a constant scale of magnification which was as nearly as possible 3000 diameters in the case of the film-preparations, 2000 diameters in the case of sections.

Our study of the development of T. lewisi in the flea was based principally upon the examination of films, made as described above, but it was found necessary also to cut sections both of the stomach and rectum of the flea. The following is an account of the technique employed by us in preparing sections of the stomach; the same applies to sections of the rectum, the only difference being that the stomachs were cut transversely, the recta longitudinally.

The stomachs of which sections were cut were taken from fleas fed eighteen, twenty-four, or thirty-six hours previously on an infected rat; the fleas themselves had been collected from the non-infected breeding-cage and kept hungry for about three days before being put on the infected rat. The stomach in each case was carefully dissected out from the flea, if possible without puncturing or injuring the stomach, in a drop of salt-solution on a slide, and then plunged into the fixative by inverting the slide in such a way that the stomach alone, all other parts of the flea having been removed, was in a hanging drop. If the stomach was ruptured or punctured in the process of extraction it was not, as a rule, preserved, except perhaps as a smear after teasing it up.

A number of different fixatives were tried, but the best results were obtained with Flemming's fluid[1] and Maier's modification of Schaudinn's fluid, and especially with the former. After Flemming the histology of the stomach is extremely good in all details; the blood fills the whole section

[1] The strong solution, made up as follows: a gramme tube of osmic acid is broken into a clean bottle, and to it is added distilled water, 50 c.c.; 1 per cent. solution of chromic acid in water, about 187·5 c.c.; and glacial acetic about 12·5 c.c.; the whole allowed to mix and dissolve.

and is not shrunk away from the wall, and the trypanosomes, both free and intracellular, are well-preserved both in structure and form, and they stain well either with iron-hæmatoxylin or Giemsa, especially the former. After Maier's fluid the histology of the stomach-tissue is not so good; the cells are shrunk and the minute structure of the nuclei is deformed. It is evident from a careful study of the preparations that the defects of Maier's fluid are due to unequal or differential penetration of its constituents; the alcohol evidently diffuses into the tissues first and produces the shrinkage and deformation of the nuclei; the sublimate does not get to the various tissue-elements until they have already been fixed in a defective manner by the alcohol. The blood-débris is also much shrunk after the Maier; while the greater part, sometimes the whole of it, contracts to form a central mass in the section, a certain amount remains usually adherent to the epithelium at the periphery, leaving an irregular empty ring-shaped space between the central and peripheral zones of the blood-débris. But to compensate for these disadvantages, the trypanosomes are extremely well-preserved and stain admirably with Giemsa's stain; some of our stomach-sections prepared in this way are as clear and demonstrative, so far as the trypanosomes are concerned, as any smear or film-preparation; in fact more so in the case of the large "spheres," which do not suffer so much from the tendency to opacity which is so disagreeable a feature in the smears. One is here confronted with the extraordinary difference, familiar to everyone who has worked at trypanosomes, between the reaction of these parasites, and that of tissue-cells, to the ordinary fixatives and stains used in cytological technique.

Whatever the fixative used, it was allowed to act for about an hour. The stomachs preserved in Flemming were well washed in tap-water and then brought up through a series of alcohols of gradually increasing strength; those preserved in Maier were transferred from it direct to 50 per cent. alcohol. In either case the objects were brought up to 90 per cent.

alcohol and there fixed on liver preparatory to being imbedded for section-cutting. Amyloid human liver was used. A moderately thin slice of a block of liver preserved in alcohol was cut by hand with a razor wetted with alcohol, and floated into a shallow glass vessel with a flat bottom, placed on the stage of the dissecting-microscope, and containing 90 per cent. alcohol to the depth of about a centimeter. The stomachs, taken up in a pipette of suitably coarse calibre, were placed on the slice of liver and carefully arranged side-by-side, their axes parallel to one another and similarly orientated, with their proventriculi all at the same level and all pointing in one direction, their pylori in the opposite direction. Then a tiny drop of glycerine and albumin solution, such as is used commonly for sticking sections on slides, was taken up on the point of a needle and caused to touch the surface of the alcohol immediately above the stomachs. The dense albumin-solution falls at once through the alcohol and spreads out over the stomachs on the liver; at the same time the glycerine is extracted and the albumin coagulated by the alcohol, with the result that the stomachs are stuck to the slice of liver. From six to nine stomachs were thus attached side-by-side on a slice of liver. As the stomachs, before being stuck on, are very liable to roll about or become shifted in position with the slightest disturbance or touch of the microscope, it was found best in practice to put them on not more than three at a time; that is to say, three stomachs having been arranged and fixed upon the liver, three more are then put on beside them. When the required number of stomachs have been stuck on, the slice of liver is trimmed with a scalpel into a rectangular form, in such a way that the longitudinal axes of the stomachs are parallel to the shorter sides of the rectangle; so that by cutting sections of the liver parallel to the longer sides of the rectangle the stomachs are all cut transversely at the same time.

We have thought it worth while to describe the method of fixing the stomachs on liver, although no novelty is

claimed for it,[1] in some detail, as it may not be familiar to some investigators working on similar objects, and because it is a procedure which saves much time and trouble. In the first place, it is much easier to imbed a relatively large block of tissue than a number of separate tiny little stomachs, and the orientation of the objects can be made much more accurate. In the second place, a great economy of labour in the section-cutting and of space in the slides and preparations is effected. To have a number of stomachs cut in the same section diminishes the labour of looking through the preparations under the microscope, and the presence in the section of the slice of liver makes it much easier to go from one section to the next under the high power. Thirdly, with a little experience the liver itself furnishes useful guidance in staining the sections, especially by the iron-hæmatoxylin method; one soon learns what degree of extraction of the stain from the liver-cells gives the best results for the trypanosomes, so that the process of differentiation can be carried out under low powers of the microscope—a great advantage. And finally, since it may be assumed that all the stomach-sections contained in one and the same microtome-section have received exactly the same treatment, it is legitimate to ascribe the very considerable differences seen in different stomachs in the same section to constitutional or functional differences in the stomachs themselves and not to varying local effects of the stain.

The stomachs, after being fixed to the liver in 90 per cent. alcohol, were imbedded in the usual way in paraffin, with a melting point of about 54° C. Methods of celloidin-imbedding were tried, but yielded no advantages to compensate for the extra trouble, especially that of extracting the celloidin from the sections—an indispensable preliminary to staining them. The best thickness for the sections of stomachs was found to be 6 μ; with less than that the trypanosomes are too

[1] One of us (E. A. M.) first became acquainted with this method in 1891 from fellow-workers in the Zoological Station at Naples, and has practised it constantly ever since.

fragmentary. The recta may with advantage be cut thinner than 6 μ, since the crithidial forms are very minute.

Various methods of staining were tried on the sections, but the results of the trials were that we kept finally in practice to two methods only, namely, iron-hæmatoxylin (Heidenhain), followed by Lichtgrün-picric in absolute alcohol as a counter-stain, and Giemsa's method. For the iron-hæmatoxylin method the sections were treated first as has been described above for the coverslip-films. The Lichtgrün-picric, which stains very rapidly, was merely washed over the sections for a moment and then washed off again with absolute alcohol. Giemsa's stain was used, according to the published prescription, as follows: The sections have their paraffin removed, and are brought down to water in the ordinary way. They are then washed in tap-water and put into dilute Lugol's solution (1 c.c. of Lugol to 25 c.c. of distilled water) for ten minutes. After this they are rinsed quickly in tap-water and put into a 0·5 per cent. watery solution of hyposulphite of soda for ten minutes. Next they are washed in a current of tap-water for five minutes or longer, and then put into the stain. The distilled water used to dilute the Giemsa-stain has to be neutralised in the way prescribed by Giemsa.[1] The sections were first placed in fairly strong stain—say, 1 drop of Giemsa to 1 c.c. of neutralised distilled-water—for about an hour, and then were left overnight in a weaker stain—1 drop of Giemsa to 4 or 5 c.c. of neutral distilled-water. The excess of stain is removed by rinsing in water, and after the excess of water has been drained off differentiation of the stain is carried out with

[1] A measured volume of the distilled water to be neutralised is taken, and to it are added a few drops of hæmatoxylin-solution (5 per cent. in distilled water), sufficient to tint it. Then a very weak solution (1 per cent. in distilled water) of potassium carbonate is added drop by drop, the water being well shaken after each drop has been added, and left for a minute or two, until the colour of the tinted water changes from yellowish-red to reddish-purple. In this way the number of drops of the carbonate-solution required for neutralising a given volume of the distilled water is known.

different strengths of acetone mixed with xylol, beginning with 95 per cent. acetone used for a very short time, in order to dehydrate the sections and extract the stain, and ending with pure xylol, after which the slides are mounted in dammar or Canada-balsam.

Of the two staining methods principally used, iron-hæmatoxylin gave admirable results after Flemming, especially for the intracellular stages; for the extracellular trypanosomes this stain is not so satisfactory, owing to the fact that the blood-débris, especially in the earlier stages of digestion, stains very intensely with it and refuses to give up the stain—at any rate not until after it has been all extracted from the cells and parasites. Consequently trypanosomes free in the blood may be entirely obscured by the opaque, deeply-stained débris, and hence quite invisible. The black stain of the blood-débris is even more intense after Maier than after Flemming; sections of stomachs fixed in Maier less than thirty-six hours after feeding are hopeless for the iron-hæmatoxylin stain, so far as the free trypanosomes are concerned, and those fixed in Flemming are not much better. By Giemsa's method, on the other hand, the trypanosomes in blood-débris are sharply differentiated and admirably shown; in the cells they are also good, better, perhaps, as "show" preparations, but not so precise in minute cytological details as by the iron-hæmatoxylin method.

To sum up the results of our experience in the technique of stomach-sections, we recommend: (1) Flemming's fluid, followed by iron-hæmatoxylin and Lichtgrün-picric; and (2) Maier's fluid, followed by Giemsa's stain. These two methods, supplementing each other, may be relied upon to reveal all essential details of the intimate life of the trypanosome and of the disturbances produced by it in the tissues of the host.

PART II.—THE DEVELOPMENT OF TRYPANOSOMA LEWISI IN THE FLEA.

(1) General Introduction.

From the results of experiments, described further below, it is shown that fleas fed on rats infected with Trypanosoma lewisi do not become infective to rats again until a period of at least five or six days has elapsed from the time that the fleas first ingested blood containing trypanosomes. From these experimental data it may be inferred that the developmental cycle of T. lewisi in the flea requires a minimum of five days for its complete course. The conclusions drawn from the experiments are confirmed by direct observation, since it is found, as will be described presently, that the little stumpy trypanosome which is the final form of the development in the flea, makes its first appearance in the rectum of the flea about five days after the development begins.

During the entire course of its development the trypanosome is confined to the alimentary canal proper of the flea, and is found in the stomach, intestine, and rectum; it is never found in the body-cavity (hæmocœle), and by a series of observations and experiments, which in our opinion are exhaustive (see below), we have convinced ourselves that the trypanosome does not penetrate into the salivary glands. It may, however, occur in the Malpighian tubules exceptionally, as the small crithidial form, characteristic of the rectal phase, attached to the wall of the tubes at or near their proximal opening into the proctodæum.

The developmental cycle can be divided conveniently into phases characteristic of the parts of the gut in which the trypanosomes are found, and we can thus distinguish a stomach-phase and a rectal phase. These distinctions are useful and natural, but their sharpness is blurred by not infrequent variations in the course of events; thus forms belonging normally to the rectal phase may sometimes be found in the

pyloric region of the stomach, though the converse case of the typical stomach-phase occurring in the rectum is not found. We may consider these phases first in their normal and typical modes of occurrence, and deal with the variations subsequently.

The stomach-phase (Pls. 36–39) is the first period of the development and is characterised by a peculiar mode of multiplication on the part of the trypanosomes, which penetrate into the epithelial cells lining the stomach and there reproduce themselves by a process of multiple fission. Hence in this period of the development free and intracellular forms can be distinguished. The stomach-phase is of short duration, perhaps in some cases lasting not more than twenty-four hours, in others two or three days, in rare cases four or even five days, but probably always terminated by the second feed of the flea, counting as the first feed that by which the flea became infected.

In the intestine the trypanosomes find, as a rule, no resting place, but merely pass through it on their way to the rectum. Hence, the forms found in the intestine are usually active, migratory forms which have completed the stomach-phase and are on their way to the rectum to initiate the rectal phase. Occasionally, however, forms similar to those characteristic of the rectal phase may be found attached to the wall of the intestine, especially near the pyloric opening.

The rectal phase (Pls. 41 and 42) consists chiefly of small, often minute individuals, which are crithidial in structure and are attached by the tip of the flagellum to the wall of the rectum, where they keep up a continual multiplication by binary fission. The crithidial form of the development takes origin in the rectum and is first established there, but may migrate forward to the pyloric region of the stomach later on. When once established in the flea, the crithidial phase endures, probably, as long as the flea lives, and thus constitutes a permanent stock of the parasite, enabling the infectivity of the flea to be maintained without renewal of the infection. From the crithidial phase arise by modification of individual

crithidial forms the small trypanosome-forms by which the infection of the rat is brought about, and which are the final forms of the developmental cycle in the flea.

By no means all the trypanosomes, however, which are taken up from the rat by the flea undergo the course of development sketched out briefly in the foregoing paragraphs. By experiment it is found that only a relatively small number of the fleas fed on infected rats become infective, apparently not more than one flea in four, on an average (see below); and these results are confirmed by direct observation. If a number of fleas are fed on a well-infected rat, trypanosomes will be found in the gut of all the fleas dissected and examined a short time after feeding; but the longer the interval between the feeding and the examination of the fleas the larger the proportion of the fleas in which the trypanosomes have disappeared or become very scanty, until finally trypanosomes will be found in but few (see Tables A and B (2) above). Probably the percentage of fleas in which the trypanosome succeeds in establishing itself permanently may be taken, on the average as about 25 per cent. (see p. 663 below). It follows that in about 75 per cent. of the fleas which digest blood containing T. lewisi the parasites die out altogether, and it is probable that in all the fleas a certain number of the ingested trypanosomes die off, since fleas that have been fed on a rat with trypanosomes swarming in the blood may exhibit a very scanty infection of the gut at any subsequent period.

From these data it is to be expected that together with developmental forms of the trypanosomes, various stages in their degeneration would also be found in the fleas, at least during the first few days after the parasites were ingested by them, and this expectation is fully realised. It is necessary, therefore, to recognise a degenerative series of forms (Pl. 43) as well as a developmental series in the gut of the flea, and to distinguish carefully the two series from one another. Any particular flea, when dissected and examined, may present an extraordinary medley of different forms of the trypanosome. To distinguish between the different forms and to refer each

form to its proper position and sequence in the series, whether developmental or degenerative, is our task, and it is no light one. When we were at an earlier stage in our investigations we did not recognise sufficiently the importance of the degenerative series, and consequently tried to interpolate degenerative forms into the developmental series, greatly to our own confusion. On the other hand it is necessary to steer very clear of a tendency to explain any form as degenerative, of which the developmental position is not immediately clear; thus we were at first inclined to regard the peculiar recurved forms in the stomach as degenerative, until we discovered the intracellular multiplication and were thereby enabled to refer the recurved forms to their true position.

In the problem of piecing together and reconstructing the sequence of the two series, developmental or degenerative, there is, to begin with, a known and fixed starting point for each, namely, the ordinary form of T. lewisi as it occurs in the blood of the rat. Further clues are obtained by linking together, through gradual transitions, the forms seen in the fleas, but more especially by the study of "time-fed" fleas, that is to say, fleas dissected and examined at known periods of time after they have been fed on the infected rat. The part of the gut in which a given form occurs is a further guide as to its significance; and all data and conclusions obtained from observation are controlled and checked by the results of experiment, especially useful in determining the final form of the development. Guided by these various considerations we have arrived at the conception, set forth below in fuller detail, of the changes undergone by the trypanosome in the flea (see especially Pl. 45 and description). In our account we describe separately the two series which we regard as developmental and degenerative respectively; but it must be pointed out that while these two series are very distinct and easily recognisable as a whole, certain forms or stages of the one series are sometimes very difficult to distinguish decisively from very similar forms belonging in reality to the other series. Consequently it is impossible to

be free from doubt, occasionally, with regard to the place to be assigned to a particular specimen or type of individual.

It remains only to be stated at this point that we adhere to the following nomenclature for the parts of the body of the trypanosome or crithidia: Blepharoplast for the basal granule of the flagellum; kinetonucleus for the smaller, trophonucleus for the larger, of the two nuclei. In order to save space we shall, however, use for the kinetonucleus the symbol *n* (plural *nn*) and for the trophonucleus the symbol *N* (plural *NN*).

(2) The Developmental Series.

(A) The Stomach-Phase.

The blood ingested by the flea passes in the first instance into the stomach, that portion of the digestive tract which is derived from the embryonic mid-gut or mesenteron, and which is lined by a layer of epithelium representing the true hypoblast or endoderm of the embryo. In the post-embryonic stages of the insect, this part of the gut is characterised by the absence of the chitinous cuticular lining secreted by the ectodermal epithelium of the parts anterior or posterior to it, namely, the stomodæum, comprising the pharynx, œsophagus, and proventriculus, and the proctodæum, comprising the intestine and rectum. The boundary between mid-gut and hind-gut is further indicated by the origin at this point of the Malpighian tubules.

In what may be called a normal feed, the flea fills the stomach and proventriculus alone. It is not an infrequent occurrence, however, for some fleas to gorge themselves to such an extent that the freshly ingested blood not only fills the stomach completely, but overflows beyond it into the intestine and rectum; we have observed this to happen most frequently in the case of female fleas, rarely in the case of males. In such cases some of the ingested trypanosomes

may be carried on at once into the proctodæal regions of the gut, but all such trypanosomes degenerate, and need not be reckoned with in the developmental series, the first phases of which take place always in the stomach alone.

(*a*) The Extracellular Trypanosomes.

The trypanosomes introduced into the stomach very soon begin to undergo changes (Pl. 36, figs. 1 and 2; Pl. 37, figs. 47 and 48). The first change is probably purely physiological, since long before any alteration is observable in form or structure these ingested trypanosomes seem to have lost their power to infect when injected subcutaneously into clean, susceptible rats (see below, p. 634). The next change observed in these ingested trypanosomes may be seen on examining microscopically the contents of a flea's stomach four to six hours after the first feed on an infected rat. A certain number of trypanosomes will then be seen to pass rapidly in a straight course across the field of the microscope with their flagella directed anteriorly. The posterior third of the body is held more or less straight and appears more rigid, as it does not share in the rapid undulations of the anterior end of the body. The movements of these trypanosomes thus contrast strongly with the sinuous, serpentine and wriggling rather than progressive movements characteristic of the trypanosomes in the blood. When not actively progressing, the trypanosomes in the stomach have a tendency to attach themselves by the tips of their flagella to pieces of débris, to the wall of the stomach, or to the surface of any other firm body. The stiffening of the trypanosome-body is probably due to increased tension of the cytoplasmic contents produced by absorption of fluid from the ingested blood as it undergoes alteration in the process of digestion. As a result of absorption or imbibition of fluid, the body of the parasite, previously more or less distinctly flattened, acquires a cylindrical and more rigid contour. If this explanation be correct, it follows that the first stimulus to developmental change is

to be ascribed to differences of osmotic tension in the fluid medium, as has been shown experimentally by Miss Robertson (1911) to be the case in the development of the trypanosomes of fishes.

In stained preparations most of the ingested trypanosomes show at first little modification from the ordinary blood-trypanosomes. In rare instances the nuclei may be approximated (Pl. 36, fig. 2). Some show a darker staining-reaction of the posterior third of the body, which appears, from the backward position of *n* in such forms, to be a sign of degeneration beginning to set in (compare Pl. 43, figs. 289, 290).

In their free active state the trypanosomes in the stomach are never found to be undergoing multiplication by any form of fission, and it is doubtful if they undergo any developmental changes further than those described above, until after they have multiplied within the cells of the lining epithelium of the stomach. The multiplication of the stomach-phase takes place solely within these cells, and although, in strict chronological order, we should now describe the intracellular stages of multiplication, it is more convenient for purposes of description to divide the stomach-phase into "free" and "intracellular" stages, and to describe all the free developmental forms before describing the intracellular multiplication.

It can be established by direct observation that the process of intracellular multiplication produces a long, free type of trypanosome which may be characterised by the term "crithidiomorphic," because while externally similar in form and movements to a large crithidial type of flagellate, it lacks, as a rule, the diagnostic structural feature of a true crithidial form, since only exceptionally is *n* found actually beside or in front of *N* (Pl. 36, figs. 3–11, Pl. 37, figs. 49–57). We shall now proceed to describe in more detail this late, free form of trypanosome. It must, of course, be understood that as several generations of the intracellular stage may follow each other in succession (see below), free and intracellular forms in all stages of development can be found

together in the same stomach. We believe, however, that the typical crithidiomorphic type of trypanosome always follows an intracellular stage, that in its less developed form it is the direct product of intracellular multiplication, and that though in this form it may again enter an epithelial cell and multiply, it is, in the more advanced form, the highest developmental type of the stomach-phase and is destined to pass down the intestine into the rectum where, after undergoing further modification, it initiates the characteristic crithidial rectal phase to be described below.

In the living condition the crithidiomorphic form progresses at a great pace in a straight line with the flagellum directed anteriorly ("mouvement en flêche"), in much the same manner as does the early stomach-form above described. It is, however, considerably longer and the posterior end is more rigid and swollen, often distinctly clubbed. Owing to the rapid motion and imperfectly straight body, the clubbed appearance is exaggerated in the living condition, but stained preparations also show that some of the trypanosomes are distinctly clubbed in shape. Like the early stomach-form, when not actively progressing the crithidiomorphic type has a strong tendency to attach itself by the tip of its flagellum to cells or débris, etc. Apart from its size the distinguishing characteristic between this and the earlier form is the marked approximation of the two nuclei, best seen in stained preparations (Pl. 36, figs. 7, 8, 10; Pl. 37, figs. 50, 51).

It has been mentioned that while, as a rule, the long, free stomach-trypanosomes have *n* behind *N*, it is found in a few cases that they have the typical crithidial structure with *n* in front *N*. We have observed altogether but three instances in which such forms occurred in sufficient abundance to make them worthy of special note. The first and most striking was the case of a flea taken from a bell-jar in which a number of fleas had been kept for some time with an infected rat, so that the length of time since the flea had ingested the parasites was not known. The body-cavity of the flea contained a cysticercoid of Hymenolepis diminuta (vide Nicoll and Minchin, 1911). The intestine of this flea showed a peculiar malformation in the form of a globular pouch-like appendix, distended with red fluid, and due apparently to an obstruc-

tion or strangulation of the intestine. The stomach, examined fresh, was seen to contain a great number of active trypanosomes, some of which were adhering together in couples, and in the intestine a clump of attached forms was seen near the origin of the Malpighian tubules.

In the preparations of the stomach of this flea a great number of trypanosomes were found showing every possible gradation of structure, from forms similar to the ordinary blood-trypanosomes to a long crithidial type with *n* far in front of *N* (Pl. 36, fig. 11 and Pl. 37, figs. 60–66). Many of these were found closely adherent in couples, just as had been seen in the fresh state, each such couple being composed of two crithidial forms in most cases, but sometimes of two ordinary forms (Pl. 36, fig. 12, and Pl. 37, figs. 67, 68). In every couple seen the two individuals appeared quite distinct and showed no signs of actual fusion; one couple was found attached tête bêche (as in Pl. 43, fig. 310). In the preparation of the rectum and intestine (preserved together) a few similar large trypaniform or crithidial individuals were seen, and also a fair number of dwarfed, degenerative forms, but no couples.

Special mention has been made of this flea because we were at first, and remained for some time, under the impression that the couples seen represented a true sexual fusion, and that we had discovered the sexual phase of the trypanosome. We have been quite unable, however, to confirm this notion or to find a similar state of things in any other flea of all those examined by us, and we now regard the state of things found by us in this particular flea as exceptional and abnormal, in relation probably to the malformations noted by us in the flea itself. It is possible that the malformed condition of the intestine prevented, to some extent, the passage onwards of the trypanosomes from the stomach, and so caused an arrest of development in the parasites, in which the tendency towards the crithidial type of structure became realised to its fullest extent. The coupling of the trypanosomes must then be regarded as agglomeration due to abnormal and unfavourable conditions, though in no case were more than two trypanosomes seen adhering together.

The second case in which the long crithidial forms were prominent was in a flea of a batch which had been fed on an infected rat three days before being examined and dissected. The fleas had been kept in an incubator at a temperature of 25° C. after the infective feed, and had not been fed again. In one of the fleas long crithidial forms with *n* in front of *N*, were fairly numerous, together with intracellular multiplicative stages, in the stomach (Pl. 37, figs. 56, 57); in the rectum one active trypanosome of the long stomach-type and a clump of degenerative forms were seen in the fresh state.

The third case to be noted was in a flea of a batch which had been fed twenty-four hours previously on an infected rat. There was nothing

special to note about this flea; the stomach contained many long active trypanosomes, with *n* and *N* closely approximated, or with *n* in front of *N* (Pl. 36, figs. 9, 10), and also some dwarfed degenerative forms, but no multiplicative stages. In the rectum a few clumps of degenerative appearance and some developmental forms were seen.

Besides these three cases which have come under our observation, in which the long crithidial type was conspicuously abundant in the stomach, we have noted the occasional occurrence of this type at various ages—twenty-four hours, forty-eight hours, and sixty hours—after the flea had fed on an infected rat. It is evident that it must be regarded as exceptional for the trypanosomes to reach the complete crithidial condition in the stomach, and that no special significance can be attributed to the crithidial form in this part of the life-cycle, although in rare instances and under special circumstances it may be abundant.

It will be clear from the foregoing remarks that we are quite unable to agree with the statements of Swellengrebel and Strickland (1910), who, having examined two fleas one day after feeding on the infected rat and five others two days after the infected feed, describe the transformation of the long crithidiomorphic type of trypanosome into the long crithidial type as the normal and usual method of development in the stomach. On the strength of somewhat more extended experience, we consider the long crithidial form to be of highly exceptional occurrence, both in the stomach and elsewhere, at so early a period of the development, as already stated; we can only explain the results of Swellengrebel and Strickland on the supposition that they were so unfortunate as to have chanced upon abnormal fleas, similar to the three cases described by us above, or that they may have regarded as crithidial forms the very commonly-occurring recurved forms, which they do not describe at all.

In view of what is known with regard to both the later development of T. lewisi in the flea and the life-cycle of other trypanosomes in their invertebrate hosts, it is evident that the crithidial type of form and structure is the principal and most characteristic phase of the development, and that there is a pronounced tendency for the trypanosome to assume crithidial characters when taken up by the flea—a tendency which asserts itself more strongly after the trypanosomes have undergone multiplication in the cells of the lining epithelium of the stomach. So long, however, as the trypanosome remains in the stomach the atavistic tendency towards

the assumption of the crithidial form $\left(\frac{n}{N}\right)$ does not normally (or, at all events, usually) get beyond the crithidiomorphic form $\left(\frac{N}{n}\right)$. Occasionally, nevertheless, the crithidial form asserts itself, as it were, even during the stomach-phase; more especially, perhaps, under the influence of any circumstances which tend to retard the development of the trypanosome and retain it in the stomach after it is ripe for passage into the proctodæum, but not infrequently even under conditions which cannot be asserted to be in any way abnormal.

(*b*) The Intracellular Multiplication of the Trypanosome.

As already stated, the multiplication never takes place in the free, active condition of the trypanosome, but only after it has penetrated into one of the large epithelial cells lining the stomach, within which it goes through a process of multiple fission to produce a number of daughter-individuals which escape from the cell and pass back into the lumen of the stomach as free trypanosomes again. The whole process of intracellular multiplication, so far as it could be made out by observation of living trypanosomes in the stomachs of freshly-dissected fleas, was described by us in our preliminary report (1911); we had not then had sufficient time or opportunity to make detailed studies, which present peculiar difficulties, of the multiplication in preserved and stained material. The ordinary smear-methods seldom permit any finer details to be made out of the trypanosomes within the cells, on account of the large size and thickness of the cells and consequent opacity of the preparation. It is only possible in smears to study the stages of multiplication set free by the rupture of the cells; but even of such specimens it is difficult to get perfectly satisfactory preparation for microscopic study of detail. With the method of fixation by vapour of osmic acid and subsequent coloration with Giemsa's stain or other

modification of the Romanowsky method of staining we have obtained occasionally very clear preparations of the later stages of the multiplication, but as a rule, the "spheres" with many nuclei take up the stain with such intensity that they become opaque masses showing nothing of the internal structure, although in the same preparations the free trypanosomes may be stained to perfection. If in such preparations the stain be cautiously extracted by means of acetone or other suitable media, it is possible to obtain specimens showing the nuclei satisfactorily, but then, as a rule, the flagella are invisible, having lost the stain completely, while the free trypanosomes or early stages of multiplication on the same slide have become mere ghosts or have vanished altogether, beyond the power of visual resuscitation by the most delicate and refined methods of microscopic illumination. Very often in such preparations only the kinetonuclei can be seen, the trophonuclei having disappeared. In the study of Romanowsky-stained preparations it was generally found necessary to begin by drawing all that could be seen, general outline, projecting flagella, in the opaque, untouched preparations of the spheres, and then to perform a number of successive operations of cautious extraction of the stain, examining the preparations after each such operation and adding to the drawing any fresh details of structure brought to light. It was difficult, however, to control the extraction of so sensitive a stain with sufficient exactness to avoid losing the whole of it in an instant. The last state of the preparation was generally one which left it useless for purposes of demonstration; always a disappointment to the microscopist and his friends. We have never succeeded in re-staining satisfactorily preparations in which the Romanowsky stain has been over-extracted.

By far the best and most instructive preparations of the intracellular multiplication were obtained in the coverslip preparations fixed in Schaudinn's or Maier's fluid and subsequently stained by the iron-hæmatoxylin method, as described above. Only in such preparations was it found possible to

control the stain so that in the largest spheres both nuclei and flagella were visible; even then, however, the trophonuclei were sometimes faint and difficult to make out clearly when the flagella were still sharp and distinct. Of one film in which the smear was thickly crowded with spheres of various sizes, some free, others still in the tissue, a very satisfactory preparation was obtained by staining with Mann's hæmatoxylin, carried out with the friendly help of the inventor of the stain himself. The result was a very good "show" preparation of the multinucleate spheres, sharp and clear, even in the thick parts of the smear, and especially suitable for moderate magnification; the flagella, however, could not be made out.

While the ordinary smear-methods presented special difficulties, very convincing and beautiful preparations of the intra-cellular phase were obtained in sections of fleas' stomachs extracted carefully from the body and preserved in various ways; a full account of the technique employed is given above. Such preparations have the immense advantage of exhibiting the exact relations of the trypanosomes to the cells; it is possible to look through every section of each series, to note every trypanosome, free or intracellular, occurring in each stomach, and to observe what each parasite was doing at the moment the stomach was preserved. On the other hand, for the study of the stages of multiplication, sections have the disadvantage that the parasites themselves are often halved or mutilated, so that any given specimen may be only a part or fragment of the whole body. Both smears and sections are therefore indispensable, and supplement each other in obtaining a complete picture of the course of events.

So much for methods and technique; we proceed now to give an account of our observations.

From Nöller's investigations on the development of T. lewisi in Ctenocephalus canis, it appears that the intracellular multiplication begins about six hours after the ingestion of the trypanosomes by the flea. In our prepara-

tions of a batch of fleas, of which the infection could not have been more than nine and a half or less than seven and a half hours old, we have found the recurved forms fairly commonly and also some of the rolled-up forms characteristic of the early intracellular stages (Text-fig. 23, p. 635). We have no other records of intracellular multiplication in our fleas earlier than twelve hours. The stages of the intracellular multiplication are to be found in all parts of the epithelium of the stomach, from close behind the proventriculus to the pylorus.

Our investigations upon the intracellular multiplication contain, unfortunately, one gap which we have been unable to fill; we have not succeeded in observing the actual penetration of the epithelial cell by the trypanosome. Nöller, however, has been so fortunate as to observe the process, and gives the following account of it. In a dog-flea which had sucked infected blood five hours and fifty-five minutes previously, he saw "a trypanosome, of which the pointed hinder end had already penetrated into an epithelial cell. The flagellum-bearing anterior end beat violently and incessantly, whereby the trypanosome penetrated further and further into the cell. After I had watched this spectacle for about five minutes the trypanosome, which had so far penetrated into the cell as far as the middle of its body, shot suddenly into the cell and stirred up the granular cell-contents by its lively movements. Since, however, the cell was torn on the opposite side, the trypanosome soon shot out of the cell again." Nöller thus confirms the suggestions we made in our preliminary report (1911) with regard to the probable method in which the penetration of the cell is affected.

We have frequently seen trypanosomes, not distinguishable in the living state from the ordinary type, singly within cells; the first time we ever discovered the trypanosomes within the cells was just such a case, a single trypanosome of quite ordinary appearance, wriggling and squirming actively in the cytoplasm of an epithelial cell, in a flea which had

been fed twelve hours previously on an infected rat. Careful examination of the cell, at different foci of the microscope, convinced us, greatly to our astonishment, that the parasite was really within the cell and not above or below it. This, and other observations, repeated subsequently upon trypanosomes of ordinary appearance, contained singly within epithelial cells, suggest that the trypanosomes in each such case had but recently penetrated into the cell; but the observation might also be interpreted to mean that the trypanosome seen was the last of a batch produced by multiple fission within the cell from which its sister-trypanosomes had already escaped. In the latter case, however, the trypanosome would probably be within a vacuole, as will be described presently.

Observation of the free trypanosomes in the living state shows that, as already stated, they are extremely active, but have a great tendency to attach themselves by the tip of the flagellum to firm objects; to the wall of the stomach, to pieces of débris, even to the glass surface of the slide or coverslip when under observation. The study of sections of the stomach confirms this observation in an unmistakable manner; many trypanosomes of the long, stiff type are seen in the sections attached to the epithelial cells by their flagella. The attachment is not, as a rule, to the outer projecting ends of the cells, but to their sides; the trypanosomes put their long flagella down between the epithelial cells and often adhere to the cell close to its base; it would appear as if the side of the cell, at least in its columnar form, is its vulnerable region. A still more striking point is that many of these trypanosomes attached to, but still quite outside the cell, have already assumed the recurved form. These observations make it very probable that in some cases the trypanosome may first attach itself to the cell by its flagellum and then bore its way into the cytoplasm in some way.

Several trypanosomes may penetrate independently into one and the same cell. We have frequently observed numbers of the parasites, from five or six up to a dozen or

more, in different stages of multiplication, side by side in a cell both in the living condition and in sections (Pl. 38, figs. 112, 113; Pl. 39, fig. 130). The parasites lie in the cytoplasm usually in a distinct vacuole, produced, apparently, by the liquefactive action of the parasite on the cytoplasm of the host-cell. The trypanosomes are nearly always in a state of movement, a point to which we shall return again. It is not infrequent to observe a number of trypanosomes in the same vacuole, wriggling actively one over the other.

The infected cell may become reduced simply to a bag containing fluid in which large numbers of trypanosomes, generally with their multiplication completed or far advanced, move actively. Such cells are found commonly in sections (Text-fig. 3); they are generally thrown off from the epithelium and lie quite free in the blood-débris, sometimes even in the centre of the lumen of the stomach; nothing remains of the cell-contents except a thin superficial layer of cytoplasm, under the cell-membrane, and the nucleus, adherent usually to the wall at some point. This condition obviously represents the last stages of the exhaustion and death of the cell, from which the trypanosomes will escape either by their own activity or by disintegration of the cell. We shall consider the effects produced by the parasites on the cells in more detail subsequently; at present it will be more convenient to confine our attention to the development of the trypanosomes themselves.

The study of the trypanosomes in the living cells, checked by the examination of preserved material, permits readily enough of the recognition of a number of well-marked stages in the process of multiplication:

(1) Trypanosomes of quite ordinary appearance, which have apparently but recently penetrated into the cell, as already described.

(2) Pear-shaped forms, with the flagellum continuing the stalk of the pear; the body of the pear is distinctly flattened, and therefore presents a contour which differs according as it is seen from the edge or from the flattened surface; as the

parasite is in constant motion within the cell it presents continually different views to the observer. The body of the parasite also shows in life incessant "metabolic" changes of form, movements of an active protoplasmic body imperfectly restrained by the thin, yielding envelope or periplast. It is very easy to see in the living condition that these pear-shaped forms are produced by the body of the trypanosome being doubled upon itself, an interpretation confirmed by the examination of preserved specimens. When the stomach is teased up and examined fresh, many of the recurved pear-shaped forms are found swimming freely, with the flagellum forward, in the salt-citrate solution used in the dissection. If such a form be watched attentively, it is often seen to uncurl itself, straightening out the body and thus passing from the pear-shaped form to that of an ordinary trypanosome. In some cases a trypanosome which had been seen to unbend itself in this manner can be observed to curl up again, while swimming freely, and thus to assume or to lose the pear-shaped form several times in succession.

In the fixed and stained preparations it is seen that the trypanosome is bent upon itself in such a way that the posterior part of the body, containing the kinetonucleus, is closely applied to the anterior half of the body. Thus a pear-shaped body results in which *N* is lodged in the thickest part of the pear, at (Pl. 36, fig. 15) or near (fig. 13) the blunt end of the body; while *n* is usually well in front of *N*, that is to say, nearer to the pointed end of the body (Pl. 37, fig. 70). In some cases, however, *n* is close beside *N* (figs. 13–17), or even, exceptionally, behind *N* (fig. 16). The variations in the positions of *n* and *N* are easily explained by their variability in this respect, already described, in the free trypanosomes, on the one hand, and on the other by variations in the exact region of the body at which the bending takes place. As a general rule, the body appears to be bent between *n* and *N*, so that *N* lies a little way from the extreme posterior end and *n* in front of it (figs. 13, 17); but the point of greatest curvature may be in the region of *N*, which is

then at the hindermost extremity of the body (fig. 15), or even in the region of *n*, which is then at the extreme blunt end (fig. 16). In exceptional cases the bending takes place in front of *N*. In all cases the flagellum runs backwards along one side of the body, round the blunt posterior end, and forward, for a variable distance, to the basal granule or true blepharoplast situated close beside *n*. Thus the course of the flagellum, as a whole, may be compared to the letter U modified by making one arm of the letter much longer than the other.

In some cases the distinction between the two limbs of the recurved body can be seen plainly in the fixed specimens (Pl. 36, fig. 15; Pl. 37, fig. 70), but in other cases no line of demarcation can be made out, and the applied portions of the body appear to have formed completely into a compact pear-shaped mass, leading on to the stage next to be described.

The recurved forms differ remarkably in size, and from a comparison of these forms with one another, with the free trypanosomes, and with later stages of the intracellular development, there can be no doubt that the initial stages of the life of the trypanosomes within the cells is accompanied by a pronounced diminution in the size of the flagellates (see especially Pl. 36, figs. 13–17, and compare them with figs. 1–10 of free trypanosomes, and figs. 21–34 of later stages, on the same plate). How this shrinkage takes place it is difficult to say; probably the cytoplasm of the flagellate gives up a large amount of watery fluid and so diminishes in bulk, while becoming at the same time correspondingly denser in texture, a change which would account for the intensity with which the intracellular forms take up the stain and the consequent opacity which they acquire, as already noted. It is necessary, however, to exercise caution in estimating the size of the forms in preparations, since there is no doubt that they vary owing to differences in fixation. Thus, Pl. 36, fig. 14 shows a specimen from the same slide as fig. 13, but the former is from a part of the film which appeared to have dried before it was exposed to the action of the osmic vapour;

its large size, light colour and the elongation of *N* are all indications that the soft body had become flattened out by being dried before fixation. The specimens may also become deformed in other ways; Pl. 37, fig. 72 is probably to be explained as representing a recurved form in which the flagellum has become torn away from the side of the body and so projects freely from the rounded posterior end.

We were at first under the impression that the pear-shaped, recurved trypanosomes found free in teased-up stomachs examined fresh were forms that had been originally intracellular and had been set free by rupture of their host-cells. As stated above, however, examination of sections proved that these recurved forms may be extracellular in occurrence, attached to the epithelial cells or even free from them. It is evident, therefore, that the recurved form is not simply an adaptation to life within the confined limits of the cell.

(3) Forms with rounded or oval body, derived from the pear-shaped recurved forms by a further contraction and rolling up of the body; these are the forms which we described in our preliminary account as "block-like" since the body often shows during life irregular contours, changing continually owing to the active metabolic movement. The flagellum, which runs in a U-shaped course in the recurved forms, acquires now an additional bend (compare especially Text-fig. 23, p. 635, *h* and *i*; it usually runs round the outer contour of the rounded body and protrudes from it to a variable extent. In some cases a very considerable length of the flagellum is free (Pl. 37, figs. 77, 78), in other cases a very little (Pl. 36, fig. 28; Pl. 37, fig. 82), while in other cases again the flagellum is simply wrapped closely round the body (Pl. 36, figs. 29, 30, 35; Pl. 37, figs. 81, 84). The extreme length of the free flagellum seen in Pl. 37, fig. 76 is possibly due in part to its having become artificially detached from the body in the process of making the smear.

The data in the foregoing paragraph have been obtained chiefly from the study of preserved specimens. In the living condition this stage appears as a small, rounded or oval body

within the cell, usually in motion and showing a distinct flagellum. Sometimes, however, these bodies are quite motionless with no flagellum visible. In our preliminary communication (1911), we were unable to decide whether a flagellum was always present, and were prepared to admit that in some cases this stage might be a non-flagellated leishmania-like form. We had observed in one case that a body which had been for some time quiet and motionless within the host-cell became suddenly active, showing a distinct flagellum. In all our permanent preparations, however, whenever flagella can be made out in the other stages or in the free trypanosomes, they can be seen to be invariably present at this stage also, and there can be no doubt that the motionless forms are those in which the flagellum is wrapped round the body. We are now convinced that non-flagellated leishmanial forms do not occur. We have the impression that the rolled-up trypanosome can wrap its flagellum round the body and pass into a resting, quiescent condition for a time, after which it can become active again by uncurling and setting free its flagellum, or at least a certain length of it, probably never quite the whole length.

The body in this stage, as in the last, is actively metabolic, with constantly changing contours in life. When the rounded forms are set free by rupture of the host-cell they swim actively in the liquid, progressing with the flagellum directed forwards; they then resemble ordinary flagellate monads, and the observer might easily have the impression that he was watching some intruding flagellate derived from contamination of the salt-solution or from some extraneous source.

In a few rare instances the rolled-up forms have been found in preparations to exhibit a central perforation or fenestration (Pl. 36, figs. 24, 30, 34), evidently produced by the trypanosome curling itself round so as to leave a central space. This condition, when it occurs, is probably quite transitory, the plastic cell-body of the trypanosome fusing into a compact lump sooner or later.

It seems probable that some of the rolled-up forms degene-

rate at this stage and are absorbed; that is, at least, the only explanation we are able to offer for such minute forms as those shown in Pl. 36, figs. 43–45, which appear to be undergoing degeneration.

The smallest of the rounded forms have each a single kinetonucleus, trophonucleus and flagellum. Now they begin to grow in size, with concomitant multiplication of their nuclei and formation of daughter-flagella. The division of these various parts appears to go on much as in other trypanosomes, independently, but more or less synchronously. The division of *n* may be slightly in advance of that of *N*, or slightly after it: thus, stages are found in which *n* and *N* appear to be both in the same stage of division (Pl. 37, fig. 79); or in which *N* appears to be in advance of *n* (Pl. 36, figs. 28 and 37); or with two distinct *nn* and *N* still in division (Pl. 37, figs. 80, 82); or finally with *n* and *N* both completely divided (Pl. 36, figs. 33, 36). The division of *n* is dependent on, or connected with, that of the basal granule or true blepharoplast, which may be regarded as representing the centriole or division-centre for *n*. The original flagellum does not divide, however, but remains attached to one of the daughter-blepharoplasts, from which it arises in close proximity to one of the daughter *nn*; and from the other blepharoplast a new flagellum grows out, at first a very fine and delicate structure and consequently very difficult to make out clearly or with certainty in the opaque body. In many cases in which *n* is divided completely no second flagellum can be seen, but it would not be safe, in view of the difficulties of technique presented by these objects, to conclude in all such cases that the formation of the new flagellum had not begun, since in other cases a very delicate line can be seen plainly growing out from the daughter-blepharoplast—that is to say, from the blepharoplast other than that from which the original flagellum arises (Pl. 37, figs. 79–81). As a rule the daughter-flagella can be made out in the preparations stained with iron-hæmatoxylin, but not in those stained by Giemsa's method; in the latter case, as already mentioned, the body

usually stains so intensely as to obscure completely the delicate daughter-flagella, which are imbedded in the mass of the cytoplasm, while the original flagellum runs for the most part on the exterior of the body; and if the stain be extracted sufficiently to make the body clear, it comes out of the growing flagella and leaves them invisible.

We may infer, therefore, that as the division of the nuclei proceeds the formation and growth of new flagella follow hard upon the division of the blepharoplast and kinetonucleus, and that a new flagellum grows out from each blepharoplast, in close proximity to *n*, quite independently of the original or parent flagellum, which remains unaltered.

As a general rule the multiplication of the nuclei begins at the rolled-up stage, but this rule is by no means invariable. Sometimes the nuclei are found to have multiplied even before the trypanosome has taken on the recurved form (Pl. 36, figs. 18–20). Such forms might possibly be specimens which, after becoming recurved, have straightened themselves out again, but their appearance is that of trypanosomes in which nuclear multiplication has begun before change of body-form. Attention must also be drawn to the peculiar elongated forms with 2 *nn* and 2 *NN*, such as Pl. 36, figs. 21–23; Pl. 37, fig. 74. At one time we were inclined to suspect that these forms, and also the unaltered trypanosomes with 2 *nn* and 2 *NN*, might be examples of fusion instead of multiplication (see below, p. 604); but we could find no definite evidence of there being fusions of two trypanosomes, and the fact that forms occur with 3 *nn* and 3 *NN* (fig. 18, pl. 36) makes it more probable that they are early stages of multiplication. On the other hand the possibility cannot be excluded that in some cases accidental and purely plastogamic (non-sexual) fusion of intracellular stages may occur, and that such fusions may explain the enormous size of some of the spheres and later stages of multiplication (Pl. 36, fig. 42).

The multiplication of the nuclei proceeds apace, and the duplication of *n* and *N* is followed by a stage in which the

body contains 3 *nn* and 3 *NN* (Pl. 36, fig. 40[1]; Pl. 37, fig. 84). This stage, which is of common occurrence, indicates that after the first division of *n* and *N* one of the daughter-nuclei in each case remains undivided, while the other divides again. This interpretation is supported by fig. 39, showing a specimen containing 3 *nn* and 2 *NN*, one of the latter being in process of division. A later stage is seen in fig. 38, in which both *n* and *N* have multiplied to four in number. The further stages of multiplication are not easy to follow in detail owing to the difficulty of making clear in one and the same specimen the *nn*, *NN*, and flagella, but from a study of various preparations there appears to be no reason to doubt that the *nn* and *NN* maintain their parallelism in division, and consequent equality of numbers, and that as the *nn*, or, to be more accurate, their blepharoplasts, divide, they continue to give rise to new flagella. Since the blepharoplasts are of different ages, as the result of successive divisions, the daughter-flagella given off from them are of different lengths; the original or parent flagellum remains, however, distinct and recognisable, both by its length, its superficial position, and the sharpness with which it stains (Pl. 37, fig. 92, etc.). In some cases the daughter-flagella also project freely from the surface of the body. This fact was observed in a living specimen, in which two small flagella were seen in addition to the principal flagellum, but it appeared to be a temporary condition, since later on only the principal flagellum could be seen, and still later that also disappeared. In one of our preparations also three daughter-flagella are seen in addition to the main flagellum, but unfortunately the specimen was so opaque that no details of internal structure, except the nuclei, could be made out.

With the growth in size and the multiplication of the nuclei the "block-like" stages pass by insensible gradations

[1] The specimen shown in fig. 40 appears to have become dried and flattened out before fixation; its size is altogether abnormal for this stage; compare fig. 38. Fig. 26, Pl. 36, also abnormally large, is from the same slide as fig. 40.

into: (4) The "spheres," a term used by us to denote the final stages of the intracellular multiplication. They appear in the fresh preparation as relatively large masses, more or less spherical in form, within the cytoplasm of the epithelial cells; they can be distinguished generally as "tailed" and "tail-less." At the very last the tail disappears in all cases, but this appendage, though commonly present in stages not full grown, sometimes cannot be made out in specimens that do not appear to be mature. A comparison of fixed preparations and a consideration of facts already discussed in describing the "block-like" stage leaves no doubt but that the tail-less spheres are derived from those earlier stages which have the original flagellum wrapped round the body, the tailed spheres from those which have the flagellum free. It is, however, evident even in the living state that the tail represents more than the original flagellum, since it is often of considerable thickness at the base and tapers gradually to a point. Examination of stained preparations shows that the tail represents in early stages the anterior end of the body, with the flagellum, of the original trypanosome (Pl. 36, figs. 37, 39; Pl. 38, fig. 110); and that in later stages the daughter-flagella may grow out parallel to the original flagellum, and so contribute to the formation of the tail (Pl. 37, fig. 86; Pl. 38, figs. 108, 111). But in other cases the anterior end of the original trypanosome may be retracted into the main mass of the sphere, round which the flagellum is wrapped more or less completely; the sphere then either has no tail (Pl. 37, figs. 90–94) or, if a tail is present, it represents the flagellum alone (figs. 95, 110).

The appearance and behaviour of the spheres in the living condition have been described by us in our preliminary communication. They are in a state of incessant movement, due both to the activity of the flagellum or tail, when present, and to internal commotions. The movements of the flagellum cause them to rotate within the cell in a jerky, irregular manner. When there are several spheres within one cell they can be seen to push and bump against

the cell-nucleus or against one another, the impact of one sphere causing a movement in another, which shows that they come into actual contact. Internal causes of movement are the metabolic form-change, already described, in the earlier stages, and movements due to the independent motility of the daughter-trypanosomes in the ripe spheres.

Even in the living condition it is not difficult to make out that a process of multiplication is going on within the sphere. First the nuclei and flagella can be seen to have multiplied, though the details of the process are naturally not clear in the living specimen. Later it is seen that the whole contents of the sphere have divided up into a number of distinct daughter-trypanosomes, which are seen writhing and twisting over each other within the enveloping periplast like a bunch of eels in a sack. The independent movements of the contained trypanosomes make their separate individuality clearer in the living state than in the preserved specimen. After the daughter-trypanosomes have become fully distinct and separate from one another a number of changes are seen in the sphere. The tail, if it was present, disappears, and the body acquires a perfectly spherical contour; this can only mean that the original flagellum, distinct from the beginning, has been drawn into the sphere and taken over by one of the sister-trypanosomes resulting from the process of multiplication. The metabolic form-changes, so marked a feature of the earlier stages, now cease, and the sphere shows only slight oscillating or trembling movements, due to the activity of the contained trypanosomes, moving restlessly within the comparatively tense envelope of the sphere, representing the periplast of the original trypanosome.

As the final moment approaches, the envelope of the sphere becomes more tense and rigid, its outline in optical section ever more nearly a perfect circle, until it bursts suddenly, setting free the contained trypanosomes. If the sphere bursts in its normal situation, that is to say within the cytoplasm of the host-cell, the liberated trypanosomes are seen moving actively for some time in the cell, from which

they escape one by one, passing out into the lumen of the stomach. But in the examination of stomachs freshly teased up, it is common to find spheres set free by the rupture of their host-cells. The spheres can then be seen to burst with such suddenness that they appear to explode; the impression given by the eye disappoints the ear, which misses the almost-expected report of the explosion; the trypanosomes set free scatter in all directions.

In one case a single cell was observed to contain three spheres, which were seen to burst successively; first one, then another, and then the third became resolved into free trypanosomes until the cell was full of active trypanosomes which appeared to be wriggling in a clear fluid. The cell retained at first its contours, but was apparently reduced to a spherical envelope, with no nucleus visible. Soon after the last sphere had become resolved the trypanosomes were seen to be escaping from a small area of the cell-wall, which then collapsed slightly; all the trypanosomes eventually found their way out at this part of the cell and swam off. In another case we timed our observations of a sphere and recorded the events in chronological order. It is not necessary to repeat the data already published, but they may be summarized as follows: A full-grown sphere was seen within a cell, moving and rotating by means of its flagellum, which was distinctly visible, and also exhibiting active metabolic form-changes; twenty-three minutes after it was first seen both the rotatory and the metabolic movements became much slower, and one minute later they ceased altogether, the alterations in the contour being slight, and due apparently to the very active movements of the contained trypanosomes; the flagellum had disappeared completely from view; the contour of the sphere then became very tense and rigid, and it burst after having been watched for twenty-eight and half minutes, or about five minutes after the disappearance from view of the flagellum and the cessation of the metabolic movements.

The trypanosomes set free are the long free stomach-type

already described, characterised by their great length, their stiff, crithidiomorphic form, and the more or less pronounced tendency to approximation of their two nuclei. The number produced in a sphere seems to vary considerably in different cases. We have attempted to count those which we have actually seen liberated within cells by bursting of spheres, and came to the conclusion that about eight was the usual number of trypanosomes produced. It is, however, very difficult to count accurately a number of trypanosomes which are at different levels in the cell, and, therefore, not all in focus at the same moment, and which at the same time are moving actively and changing their position. Moreover, the full-grown, tense spheres, which can be observed to burst, differ so greatly in size that there can be no doubt that the contained trypanosomes must vary considerably in number, and that if the average capacity of a sphere be eight trypanosomes, some spheres must liberate not more than four, others, perhaps twelve or sixteen, when they burst. These conclusions are confirmed by a study of fixed preparations; in the large spheres we find 8 (Pl. 37, fig. 86), 10 (Pl. 37, fig. 96), 13 (Pl. 36, fig. 41), and 14 or 15 (Pl. 37, fig. 94, and Pl. 36, fig. 42) *nn* and *NN*. From the results of counting the nuclei in a number of preparations, the most frequent number of *nn* in the largest spheres would appear to be 10 *nn*; the number of *NN* cannot always be made out, but where they can be seen their numbers are equal to, or slightly less than, those of the *nn*, indicating that the *nn* divide a little in advance of the *NN*. On the other hand, some spheres with but few nuclei appear to be nearly mature, having daughter-flagella so long that the nuclear division must have become much retarded, or perhaps ended altogether (Pl. 36, fig. 40, and Pl. 37, fig. 95). The permanent preparations confirm, therefore, the conclusion that while the number of trypanosomes produced is commonly eight or ten, it may be less, or as many as fourteen or more. The intracellular growth and multiplication of the trypanosome must depend on an interaction between the host-cell and

parasite, each a variable factor, and it is, therefore, not surprising if the result varies in different cases. Moreover, when a cell contains several parasites in different stages of multiplication, the cell may become exhausted before the younger individuals have time to grow to their full size.

The length of time required for a complete generation of intracellular multiplication is a point we have been unable to determine, as it is impossible to keep the living object alive under observation long enough. Moreover, the living trypanosomes in teased-up stomachs soon become abnormal, however carefully sealed up between slide and cover-slip, so that no data derived from direct observation of this kind could be entirely reliable. Until physicists and opticians have invented some method whereby the epithelial cells of the flea's stomach can be focussed under high magnification without dissecting or injuring the flea, it will not be possible to give a direct answer to such questions. Our observations cited above show that barely five minutes are required for a sphere to burst after the multiplication is complete, but that gives no clue to the length of time required for the growth and multiplication of the original trypanosome after it has penetrated the cell. From the fact, already noted, that the earliest intracellular stages were found by us about eight hours, and the earliest trypanosomes of the long stomach-type about twelve hours, after the flea had fed on an infected rat, it might be inferred that an intracellular generation required about four hours, but this computation cannot claim to possess any exactness.

It is also not possible to state how many successive generations of multiplication take place in the flea's stomach. Nöller states that at least two such generations, probably more, succeed each other. From the fact (to be described in more detail below) that the stomach-phase may be ended in some cases in eighteen hours, and in other cases may persist for four or even five days in unfed fleas (see above), it is evident that the number of generations of intracellular multiplication in the stomach must be a highly variable quantity.

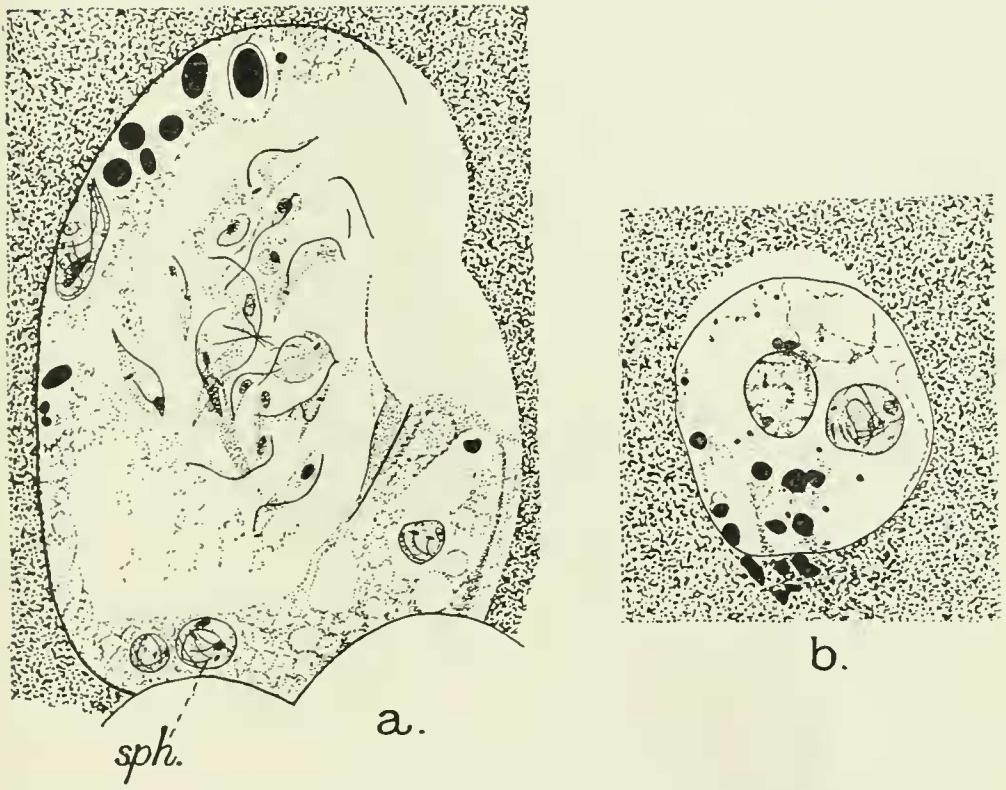
The foregoing account of the stomach-phase has been based almost entirely upon the study of the trypanosomes in the living condition and in smears. It was, in fact, all written, except for a few subsequent additions, before we had studied any sections. It remains now to supplement the description given above with the additional data furnished by the study of sections of the stomach, whereby a much more exact idea can be obtained of the relations of the trypanosome to the epithelium of the stomach.

When sections through a well-infected stomach are examined the trypanosomes are found usually to be both intracellular and extracellular in occurrence; that is to say, both free in the lumen of the stomach and contained within the cells of the epithelium. The extracellular trypanosomes occupy two different positions, speaking generally: they may be scattered throughout the blood-débris, including its most axial region, or they may be found only along the periphery of the sections, close to the epithelium and very often attached to it by the tip of the flagellum.

There can be no doubt that the trypanosomes found scattered through the central part of the blood-débris are for the most part forms in process of migration towards the rectum, their stomach-phase having been completed, but not all of them are to be interpreted in this way, since in many cases they are seen to be in clumps connected together by the tips of their flagella, and are obviously degenerative forms. In many cases also an infected epithelial cell is thrown off into the lumen, and may be seen there, crammed with trypanosomes, the product of the intracellular multiplication, which break out of the exhausted cell, and may possibly find their way into other epithelial cells again. The cell itself may become reduced to a mere sac containing fluid, in which a huge number of trypanosomes are squirming over one another (Text-fig. 3). If any of the protoplasm of the cell still survives it may contain a few spheres and other multiplication-stages. But that the forms free in the débris are for the most part migratory forms is indicated

by the fact that they occur with greater frequency towards the pyloric end of the stomach and sometimes only in this region, which may swarm with them, and in such cases they are always to be found in the intestine also.

TEXT-FIG. 3.



Exhausted cells containing intracellular stages of the trypanosome, from sections through the stomach of a flea thirty-six hours after the infective feed, $\times \frac{4000}{3}$. (*a*) A cell in process of being pushed off from the epithelium; the indented outline of the lower edge shows where it was in contact with epithelial cells in situ. The cell is reduced for the most part to a sac of fluid, in which trypanosomes are swimming, but round the edge some of the cytoplasm still remains, and in it are seen a few multiplication-stages (sph.), and also a few coarse black grains of fatty nature. The nucleus of the cell comes in another section. (*b*) A cell completely detached from the epithelium, the cytoplasm reduced to a few irregular strands and containing: (1) the nucleus at the centre; (2) a sphere to the right of it; (3) some coarse fat-grains, some of which have been extruded into the blood-débris.

On the other hand the trypanosomes that occur in close proximity to the epithelium are for the most part forms about to attack it, as shown by the fact that many of them are actually attached to the cells, and also by the frequency, it might almost be said the predominance, of the recurved form in this situation; at any rate, most, though by no means all, of those that are actually attached to the cells are recurved (Pl. 38, figs. 104, 105; Pl. 39, figs. 120–124). A certain number of recurved forms are also found, however, quite free from the epithelium, though close to it (Pl. 39, figs. 118, 119). The question at once suggests itself whether that represents the state of things as it was in life. It is impossible to extract a stomach from a flea without doing a certain amount of violence to it; and it is quite conceivable that the stresses and strains to which the wall of the stomach is subjected in pulling it out of the flea's carcase might well cause trypanosomes, previously attached, to become detached from the cells. We can but take the evidence as it is presented to us in our preparations, however, where we find that some of the attached trypanosomes are recurved and some not, and that some of the recurved forms are quite free from, though always close to, the epithelium. It would follow from these observations that in some cases the trypanosome assumes the recurved form after, in other cases before, attaching itself to the epithelial cell.

The attachment of the trypanosomes to the cells is peculiar; they are seldom attached to the extreme apex of the epithelial cell, but usually to its side (Pl. 39, figs. 121–124). The long flagellum runs down in the interspaces between two adjacent cells, and its extremity is often seen to be adherent to one of the cells close to the isthmus connecting it to its neighbour; the body of the trypanosomes usually projects into the lumen of the stomach above the general level of the epithelium, but sometimes the whole body is deep in between the cells. Sometimes two or three trypanosomes are attached side by side in such a situation. These statements apply to the usual columnar form of the epithelium with deep spaces inter-

vening between the cells; when the cell is flattened the trypanosomes may be attached at any point, and in the case of the young cells, which have but recently been produced from the epithelial buds and in which each cell is in contact with its neighbours along its side, without interspaces, the trypanosomes are attached to the convex apical region of the cell (Pl. 38, fig. 104), which may even contain early intracellular stages (Pl. 39, fig. 131).

As stated above, we have never been able to observe in the living state the actual penetration of the epithelial cell by the trypanosome; and in our sections we have searched in vain for appearances which could be interpreted as trypanosomes caught in the act of penetration. We have not found any trypanosomes half in and half out of the cell, from which it may be inferred, probably, that the actual penetration is effected very rapidly. But attention must be drawn here to a condition which is probably of significance in connection with this point. Trypanosomes of recurved type are found with the tip of the flagellum attached close to the base of the cell and with the rest of the body so closely applied to the side of the cell that careful focussing is necessary to determine that the trypanosome is still bodily outside the border of the cell and has not invaded it. Such appearances (Pl. 39, figs. 122, 123), suggests powerfully that the parasite, after attaching itself by the flagellum, forces its body into the cell. But the imagination must not be given too loose a rein when interpreting in terms of living activity the appearances seen in the dead fixed preparations.

The next stage is a recurved trypanosome within the cell (Pl. 38, fig. 107). Trypanosomes of the ordinary form (i. e. not recurved) are often seen in cells, but it is doubtful whether these represent individuals that have but recently penetrated the cell; if so, they might be forms which, after having been recurved, have temporarily straightened themselves out again, but it is on the whole more probable that they are trypanosomes which have been produced by the disruption of a sphere and which are about to leave the cell. In

support of the latter conclusion is the fact that we have seen trypanosomes of ordinary type only in cells that appeared more or less exhausted and liquefied, and especially often in detached cells. But, even if such forms represent recent arrivals in the cell, it is certain that they soon take on the recurved form. Consequently the recurved form may be taken in any case as the starting point of the intracellular development.

The growth of the recurved form into the sphere, the multiplication of its nuclei and flagella, and its ultimate fission to produce a bunch of trypanosomes, have been described in detail above, and only a few points in the development of the intracellular phase need be noted here. In the first place, in all preparations satisfactorily stained, it is seen clearly that flagella are present throughout, and that non-flagellated or leishmanial forms do not occur. This is so in all preparations stained with iron-hæmatoxylin and not over-extracted. In preparations stained by Giemsa's method the flagella are sometimes not to be seen, but in successful preparations they are exceedingly clear and sharp; it is always, however, one thing or the other, that is to say, we never find in one and the same preparation some parasites showing flagella and others none; either the flagella are invisible throughout the preparation, or are to be seen in every trypanosome, whether inside or outside the cells.

In sections the parasites are naturally very often in a mutilated condition, especially the large spheres, which may go through two or more sections. Consequently, it is not possible to be quite certain always whether a given sphere is tailed or not. But if the tail happens to lie in the plane of the section it may be shown very clearly, and then it can be seen that in some cases the tail is simply the original flagellum of the parent-trypanosome, projecting freely, while in other cases the tail includes the original flagellum with a prolongation of the trypanosome-body into which daughter-flagella may grow out, alongside of the parent-flagellum (Pl. 38, figs. 108, 110). In some cases, especially when the

host-cell is degenerating and breaking up, the flagellum of a sphere may project out from the cell (Pl. 39, fig. 132).

In concluding the description of the intracellular multiplication, we note that Chatton and Leger (1912), have described what they term a process of "agglutination and cytolysis" in Leptomonas drosophilæ, and suggest that the intracellular multiplication seen by us in the case of Trypanosoma lewisi in the stomach of the flea may be a phenomenon of the same order as that observed by them in Leptomonas drosophilæ. We cannot accept for a moment the suggestion that the behaviour of T. lewisi in the flea's stomach is anything but a quite normal process of multiplication. It is not incumbent upon us to criticise the statements of the authors, otherwise we might be tempted to inquire why they should think it necessary to interpret what they have seen in their Leptomonas as agglutination and cytolysis rather than as multiplication; but however legitimate such an interpretation may be for L. drosophilæ, we cannot admit it for the similar phenomena observed by us in T. lewisi.

Appendices to the Description of the Stomach-phase.

Having given in the foregoing pages an account of the intracellular multiplication of the trypanosomes in the stomach, it remains to discuss the relations of parasite to host and vice versâ. Under this theme are comprised (1) the frequency of occurrence of the intracellular multiplication in fleas exposed to infection, (2) the type of epithelial cell attacked by the parasite, (3) the relation of the intracellular stages to the infected cell, (4) the effects of the parasites on the cells, and (5) the relation of the infection as a whole to the stomach. The first of these problems is a statistical one, and is best studied in fresh or preserved film-preparations; the remaining four questions are best studied in serial questions.

(1) The Occurrence of the Intracellular Multiplication.

The following is a summary of our observations on the occurrence of the intracellular multiplication from twelve hours onwards, not counting those fleas that were used for sections:

At 12 hours approximately, after the fleas had fed on an infected

rat, intracellular multiplication was found in one flea out of ten examined:

At 15 hours, in	1 flea	out of	15.
At 18 ,,	2 fleas	,,	15.
At 20 ,,	1 flea	,,	2.
At 24 ,,	40 fleas	,,	240.
At 36 ,,	2 ,,	,,	5.
At 48 ,,	5 ,,	,,	20.
At 60 ,,	none	,,	22.

At three, four and five days after infection, we only found trypanosomes in the stomachs of fleas that had been kept isolated and starved; in those that had been allowed to feed again on a clean rat no trypanosomes of any kind, free or intracellular, were found in the stomachs. Counting, therefore, only starved fleas, we found intracellular stages:

At 3 days, in	5 fleas	out of	16.
At $3\frac{1}{2}$,,	1 flea	,,	7.
At 4 ,,	2 fleas	,,	13.

Later than five days we have never found them.[1]

The total of all these observations is that we have found intracellular stages of multiplication in the stomachs of sixty out of 365 fleas examined from twelve hours to four days after infection, or approximately 16·5 per cent. There is no doubt, however, that this figure falls below the actual percentage of fleas in which the trypanosomes succeed in establishing themselves in the stomach and passing through their intracellular phase when ingested by the flea, for at least two reasons. In the first place, many of the 240 fleas dissected at twenty-four hours after the infective feed were examined very hurriedly and cursorily, the object being to find fleas in which the intracellular stages were swarming, for the purpose of making permanent preparations of these stages, and when they were not found quickly in the teased-up stomachs examined in the fresh state, the search was abandoned; consequently in many cases in which the intracellular stages were scanty they may well have been overlooked, and would probably have been found after more prolonged search.[2] In the second place, as regards the fleas examined

[1] Since this page was written we have found intracellular stages in a flea which had been fed on an infected rat five days previously, and since then had been kept starved.

[2] Compare especially the figures given below (Text-figs. 4–12) of stomachs reconstructed from serial sections; it will be seen that there may be instances in which only one or two small patches of epithelium are infected, and in such cases it would be a lucky accident if the infection were discovered in looking at the freshly teased-up stomachs, even after a prolonged search.

at later periods than twenty-four hours, it is highly probable that in many of these the intracellular multiplication may have been completed and this stage passed. In one of our series of sections of a stomach preserved thirty-six hours after infection the stomach itself contained no trypanosomes of any kind, but two clumps of typical crithidial forms were found attached in the intestine behind the pylorus (Pl. 42, fig. 273); in this case the stomach-phase must have been over, assuming that it is a necessary and essential part of the developmental cycle. In Table M it will be seen that in several cases fleas dissected at two or two and half days after the infective feed had no trypanosomes in the stomach but developmental crithidial forms in the rectum. The absence of intracellular multiplicative forms in the stomach of a flea examined more than twenty-four hours after the infective feed is, therefore, no proof whatever that this phase has not been passed through in that flea.

When due weight is given to both these considerations, namely the probability that scanty infections may be overlooked in hasty examinations of stomachs in the fresh conditions, and the fact that the stomach-phase may be passed through rapidly and ended very soon, there can be no doubt that the true percentage of fleas in which it must take place must be considerably higher than the figure, 16·5, derived from actual observation. We should probably not be far wrong in putting it above 20, a result agreeing fairly closely with the percentage of fleas found by direct experiment to become infective after having fed on an infected rat (see below, p. 663), and thus supporting our belief that the intracellular multiplication is necessary for the trypanosome to establish itself in the flea, and is an indispensable part of the life-cycle ; a belief which is not, however, under the circumstances, capable of direct proof or verification.

It is at least evident from the figures given that the investigator must not expect to find the intracellular multiplication in more than a fraction (1/6 or 1/5) of the number of fleas examined which have fed but once on an infected rat ; and that he is most likely to find them from eighteen to thirty-six hours after the trypanosomes have been ingested by the flea.

(2) The Type of Cell attacked by the Trypanosome.

With regard to this point we have to note, in the first place, that we have never seen intracellular stages of the trypanosomes in the cells of the epithelial crypts. In this we are in agreement with Nöller (1912), who has also studied sections of the flea's stomach. Extracellular trypanosomes are sometimes seen attached to the exterior of cells which are passing out from the crypt, but never inside any cell which is not definitely a part of the general epithelium. It is possible that

the separation which, as described above (p. 494), takes place between the epithelial cells until they are connected only by the isthmus at the base, makes it easier for the trypanosomes to penetrate into them, since the appearances seen in sections suggest that the attack is usually made on the side of the cell (Pl. 39, figs. 120–124); the occasional, though rare occurrence, however, of intracellular stages in quite young cells (Pl. 39, fig. 131), shows that the trypanosomes can penetrate into epithelial cells before the separation between them has developed.

The degenerated epithelium, full of fatty deposits which blacken after treatment with osmic mixtures, is also not attacked by the trypanosomes. It is true that intracellular stages may sometimes be found in a degenerated cell, but in such cases the cell has been thrown off from the epithelium and the parasites are in the condition of large spheres ripe for breaking up into trypanosomes (Pl. 39, fig. 135); or of masses and clumps of trypanosomes that have evidently been liberated by the recent disruption of a sphere (Pl. 38, fig. 114). It may be supposed that in these cases the cell was attacked by the trypanosomes before degeneration had set in; the process of degeneration may have been hastened by the action of the parasites.

The trypanosomes attack by preference the fully-formed, but still young and vigorous cells, which may contain granules of the normal type and even yellow bodies, but no fatty deposits; cells which may be well characterised as adolescent in type, and which stain a clear, light-grey with iron-hæmatoxylin after Flemming-fixation. It is in such cells that the earlier stages of the intracellular multiplication are to be found in a flourishing condition and often in considerable numbers; but if the trypanosomes are numerous the cell soon becomes exhausted.

(3) The Relation of the Trypanosomes to the Cells.

The intracellular parasites are found most frequently in the apical expanded region of the cell, where the cytoplasm is usually of a loose spongy texture (Pl. 39, fig. 126). Sometimes, however, the trypanosomes are seen below the nucleus and occasionally there may be parasites lodged above and below the nucleus in the same cell (Pl. 39, fig. 134). Since the position of the nucleus in the cell is subject, as has been pointed out above, to variation, no special significance attaches to this point. It may be noted, however, that when a sphere is situated, as regards the principal mass of its body, above the nucleus, the tail of the sphere may run down to the base of the cell (Pl. 38, fig. 108).

In epithelium of the flattened form the intracellular parasites are lodged beside the nucleus, which is often pushed to one side of the cell.

(4) The Effects of the Trypanosomes on the Epithelial Cells.

In describing the pathological effects of the trypanosomes, we may begin with the cases where a cell is attacked by a few, not more than two or three, parasites, and has been but little affected by them (Pl. 39, figs. 126, 127). It is seen that the parasite is lodged in a distinct space or vacuole in the cytoplasm, a vacuole which is not to be interpreted as an artefact due to shrinkage from the action of reagents, but as produced by the liquefactive or absortive action of the parasite on the cytoplasm, since the vacuoles containing the intracellular parasites can be seen very clearly in the living cell. Similar effects produced by Toxoplasma gondii in the peritoneal cells of the mouse have been described by Miss Pixell.[1] In the living condition, as stated above, the trypanosomes move freely by means of their flagella in the liquefied cytoplasm, and when there are many parasites in the same cell they can be seen to jostle one another and even to bump against the cell-nucleus. Owing to the liquefaction of its cytoplasm the cell becomes empty and exhausted, and in its final stage is reduced to the condition of a sack, containing fluid, which may be crammed with trypanosomes produced by the process of multiplication within the cell; remnants of the cytoplasm may persist on the wall of the sac, and the cell-nucleus is also to be found at some point, generally adherent to the wall. Often these exhausted cells contain fatty deposits and also the brownish-yellow bodies described above.

The effect produced on the parasitised cell as a whole is a more or less distinct hypertrophy, more obvious when the contained parasites are present in large numbers (Pl. 38, fig. 114). Then the cell often becomes very much enlarged, but this enlargement appears to be brought about in many cases not solely by the hypertrophy of the individual epithelial cell, but also by the fusion of distinct cells; at least this is the only way in which we are able to interpret the large multinucleate cells, containing numerous parasites in all stages of their multiplication, that are frequently to be found. Mitoses, or any other forms of nuclear multiplication are never seen in the epithelium, and the very large size of these multinucleate cells (Pl. 39, fig. 128; compare Pl. 44, fig. 313), certainly indicates cell-fusion having taken place. We have also seen multinucleate epithelial cells not containing parasites: but such cases might very well be those in which the intracellular multiplication was ended and the daughter-trypanosomes produced had escaped from the cell.

As the cells are exhausted and destroyed by the parasites there is a pronounced tendency for them to be thrown off from the epithelium.

[1] 'Proc. Roy. Soc.,' (b), lxxxvii, p. 73, (Pl. ix, figs. 3–6).

In the sections infected patches of epithelium are of frequent occurrence, in which the infected cells are seen bulging far out from the general level of the epithelium into the lumen of the stomach, giving sometimes an appearance like a bunch of grapes (Pl. 44, figs. 313 and 315). In such protuberances every cell, as a rule, contains numerous parasites in all possible stages of multiplication, in progress or completed. In one of our series there is a stomach in which no parasites are to be found within the epithelial cells in situ in any part of the stomach-wall, but in the fore part of the stomach there are very numerous intracellular parasites, all contained in cells completely detached from the epithelium. On the other hand very large patches of epithelium may be found infected without the cell being thrown off; in one of our series there is a stomach, which, in one part shows infected cells the whole way round the section, except in the epithelial crypts (Text-fig. 9), but there is very little detachment of epithelial cells. It is evident that the expulsion of the infected cells from the epithelium is a measure partly of the intensity of the infection, i. e. the number of parasites contained in each cell, and partly of the length of time during which the parasites have been acting on the cells; it is not till the cells are becoming exhausted and incapable that they are ejected from the epithelium.

From a study of the extracellular trypanosomes, which occur in close proximity to the epithelium, and more especially those actually attached to it, it is evident that the extent of the epithelial areas simultaneously attacked may vary very greatly in different cases. Sometimes only an attached trypanosome is to be seen here and there, scarcely so much as one in each consecutive section on the average; this corresponds to the frequent occurrence of solitary epithelial cells, or very small groups of them, containing parasites. Sometimes, on the contrary, all the epithelium on one side of many consecutive sections will be seen to have trypanosomes adherent to it, while on the other side of the same sections not a trypanosome is to be seen (compare Text-figs. 4–12). What determines the extent and distribution of the attacks it is impossible to say, but at least one necessity is probably the presence of regenerated "adolescent" epithelium.

It should be mentioned finally that in one of our series there is a stomach which in its hinder region is almost entirely denuded of epithelium. Vast numbers of trypanosomes are seen in all parts of the stomach-lumen, but swarming most thickly near the wall, on which only the epithelial crypts remain intact. Remnants of the general epithelium occur here and there in the form of broken-down cells containing the final stages of the intracellular multiplication, but for the most part the epithelium is gone altogether. In the anterior region of this stomach, trypanosomes are less abundant and the epithelium is in process of regeneration. It is surely a fortunate circumstance for the

insect host that its epithelial crypts of regeneration are immune to the attacks of the trypanosomes; were it not so, it is hardly credible that the flea could survive the extensive destruction of the epithelium that may occur with an intense infection.

(5) The Relation of the Trypanosome-Infection, as a Whole, to the Stomach of the Flea.

This point can best be made clear by describing a few examples, which will give a more graphic picture of the variations seen in different fleas than can be obtained from a general description. It must be stated, in the first place, that, as already pointed out above, in stomachs of fleas preserved eighteen, twenty-four, or thirty-six hours after having been fed on a well-infected rat, the trypanosomes may have entirely disappeared, having been digested with the blood and failed to establish themselves. Consequently, in many of our series of sections, some or all of the stomachs contain no trypanosomes at all. On account of the great expenditure of labour and time involved in searching through a complete series of sections of a stomach we have not attempted to compile any statistics of the numerical proportion of infected to non-infected stomachs, as we have done in the case of stomachs teased up and examined fresh, the latter being a method much less laborious, though not so exact, for determining whether a stomach contains trypanosomes or not.

In the examples we are about to give we deal only with those stomachs in which trypanosomes have been found, and in order to exhibit at a glance the state of things in each stomach we have made reconstructions of a certain number of stomachs in the following manner:

(a) To show the distribution of the cells containing intracellular stages, the stomach is imagined as cut open from the intestine to the proventriculus along a line which corresponds to the southernmost (lowest) point in the transverse sections, but which is a purely arbitrary line so far as the flea is concerned and does not correspond to any definite anatomical plane of the insect. The wall of the stomach, supposed to have been cut open along this line, is further imagined as laid out flat. Of each stomach reconstructed in this way a diagrammatic sketch was made on paper ruled in millimeter squares to a definite scale, namely, 1 mm. to each transverse section of 6 μ in thickness, and in the sketch three meridians are put in with dotted lines, the middle one to represent the northernmost (uppermost) point of the sections, while the meridians to the left and right represent respectively the extreme western (left-hand) and eastern (right-hand) points in the section. After making a diagrammatic sketch in this manner the series of sections was searched through and all infected cells were mapped out

in the reconstructions, being represented by little circles placed in the line corresponding to the number of the section, and in the relation to the meridians that indicate its position in the section. For instance, if an infected cell is found in the twenty-seventh section, to the north-east of the section, the circle representing it is put into the diagram 27 mm. from the anterior end of the stomach and midway between the meridians N. and E.

In this way a graphic representation, accurate to scale in the longitudinal direction, is obtained of the distribution of the infected cells in the epithelium. It is important to remember that each little circle represents not merely a single intracellular stage of the trypanosome, but an infected cell which may contain many such stages.

(b) To show the distribution of the extracellular trypanosomes the diagram is supposed to represent the unopened stomach, and the trypanosomes that are in close proximity, or attached, to the epithelium are put in along the sides, while those scattered free in the débris are represented dispersed through the diagram, in the position corresponding to the number of the section in which they occur; when very numerous, however, it is impossible to represent them accurately, and they are merely crowded in as thickly as possible in the diagram.

It is very important that the reader should understand clearly that in each of our diagrammatic reconstructions of the stomachs two distinct conventions are combined, as it were superposed one on the other. Originally our intention was to have given two diagrams for each stomach, one reconstructed according to method (a) to show the distribution of the intracellular parasites, the other according to method (b), indicating the occurrence of the extracellular trypanosomes. To have given two diagrams for each stomach, however, would not only have taken up much space, but would not have shown the state of affairs so graphically as when the two diagrams are combined into one and no confusion can arise if it is clearly understood that two different methods of reconstruction are combined in each figure. The fact that the attached trypanosomes are represented adherent to the sides of the diagram, for example, does not mean that they are attached only along the southern meridian; they are attached at any point in the section, but had they been indicated in the diagram in the exact meridian in which they occur the eye would not have distinguished them from the free forms.[1] To indicate the exact position of the attached trypano-

[1] It was suggested by a friend that the attached trypanosomes might have been distinguished from those that are free by drawing at one end of them a little transverse line or semicircle, to indicate the attachment to the cell; but where the trypanosomes are thickly crowded, as in Text-fig. 10, it would have been impossible to distinguish clearly the free and the attached forms in this way.

somes it would have been necessary to have shown the attached and the free trypanosomes in separate diagrams; either to have put in the attached forms in the diagram showing the infected cells, according to method (a), or to have constructed three diagrams showing the infected cells, the attached trypanosomes, and the free trypanosomes on each diagram separately.

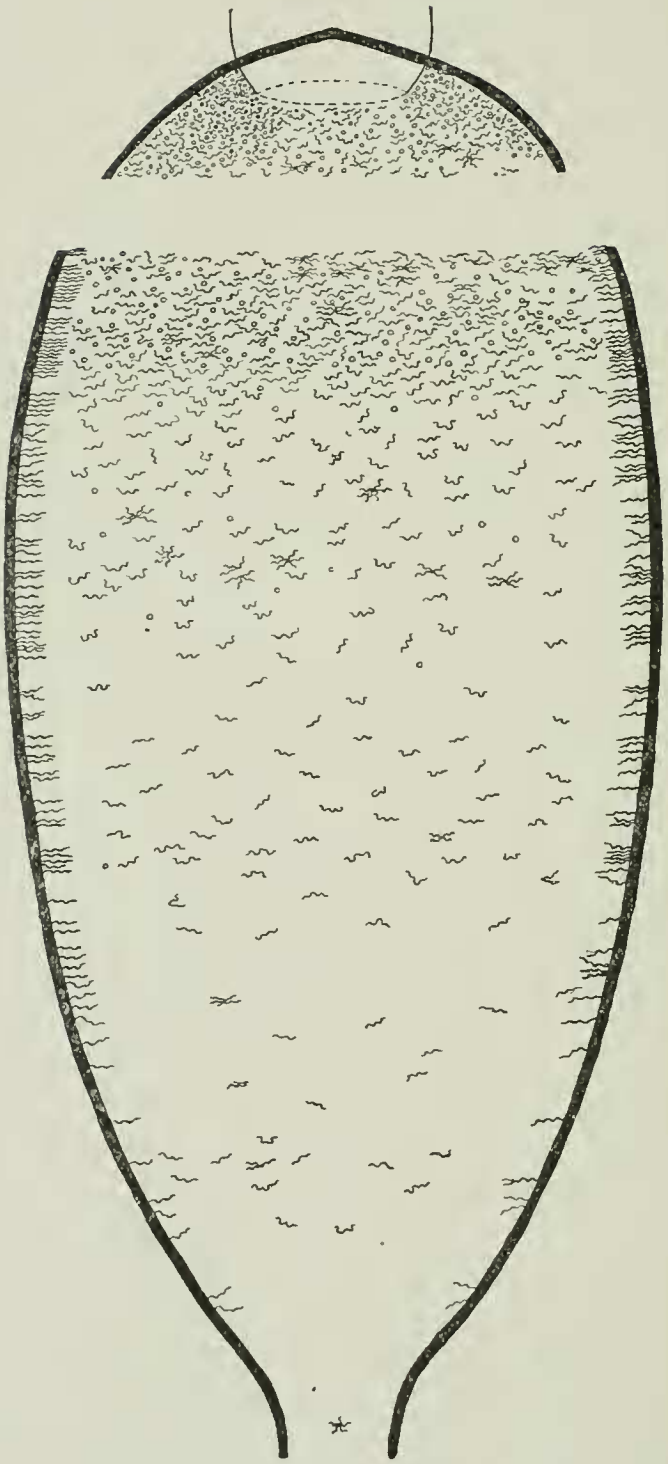
The reconstructions were made, as has been stated, on a definite scale as regards the length of the stomach, namely, on the scale of 1 mm. to 6 μ, that is to say at a magnification of $\frac{1000}{6}$, or 167 approximately. In the reproduction, however, the diagrams have been reduced to two-thirds of their original size, and appear, therefore, at a magnification of $\frac{2000}{18}$, or 111 approximately, with the exception of figs. 3 and 11, which it has been necessary to reduce to one-half on account of the very large size of the stomachs, and which, therefore, appear at a magnification of about 83·5, while fig. 9 has been reduced to one-third. The diagrams show the great variations in the size of the flea's stomach. The small stomachs are males, the large ones females. The number of sections in a series cut through a small stomach was generally about 125, in a large stomach from about 220 to 300. This corresponds in a remarkable way to some observations we had made and recorded long previously to cutting the sections—on the feeding of the fleas. We found that a male flea took about a minute and a quarter to fill its stomach, a female about two minutes and a quarter.

The stomachs reconstructed by us were all from fleas fed twenty-four or thirty-six hours before being dissected and preserved. We will begin with the fleas of thirty-six hours, because we possess a very good series of this period, which has been studied very carefully by us, and which, having been preserved in Flemming's fluid and stained with iron-hæmatoxylin, shows the state of the epithelium and the stomach-contents particularly well.

(a) Thirty-six hours after feeding.—A batch of nine stomachs, all preserved at the same time, stuck on the same piece of liver and cut in the same block of paraffin. Owing to an accident to the block the two stomachs near one end of the liver were damaged; the anterior half of one of these stomachs and a small part of the anterior end of the other were broken away. In what remained of these two stomachs no trypanosomes of any kind were found, and the same was the case with one of the other stomachs in this series. There remain six stomachs to be described.

(1) (Text-fig. 4). The stomach is a large one, going through about 260 sections, corresponding to a length of about 1·56 mm., exclusive of the proventriculus. The blood-débris is large in amount and stained

TEXT-FIG. 4.



Reconstruction of a stomach in the manner described in the text, to show the distribution of the intracellular and extracellular trypanosomes. The portion left blank is where some of the sections were accidentally destroyed. No meridians have been put in because, in this instance, all the intracellular trypanosomes were in detached cells. 36 hours. Magnified about 83·5.

yellow. The epithelium is for the most part flattened and very black, especially at the anterior end, where also there are very many detached cells. There are also a great many yellow necrosed cells, either in the form of extensive patches in the epithelium in situ, or of detached cells loose in the blood-débris.

Extracellular trypanosomes abundant, especially round the detached cells in the anterior region. In the posterior half of the stomach they are much fewer, and almost all peripheral in position or attached to the epithelium.

Intracellular trypanosomes very abundant in the anterior fourth, but only in detached cells, none in the epithelial cells in situ. Passing backwards they become scarcer, and in the posterior half of the stomach none are found.

Since the intracellular parasites in this stomach are found only in detached cells, the meridians have been omitted in the reconstruction, it being impossible to refer the infected cells to their proper position in the stomach-wall.

Interpretation.—This is a stomach in which the epithelium is in the final stages of degeneration, especially anteriorly, but regeneration has not begun. There has been an extensive intracellular infection in the most anterior region, represented now by advanced stages in the detached epithelial cells, and swarms of free trypanosomes are attaching themselves in preparation for a fresh attack. There are also clumps of degenerative forms free in the blood.

(2) The stomach goes through about 220 sections corresponding to a length of 1·32 mm. Blood-débris in moderate quantity, stained yellow; epithelium clear, granular, containing many grains and pseudospheres. No trypanosomes in any stage found in the stomach, but in the intestine, behind the pylorus, in a large clump of attached crithidial forms.

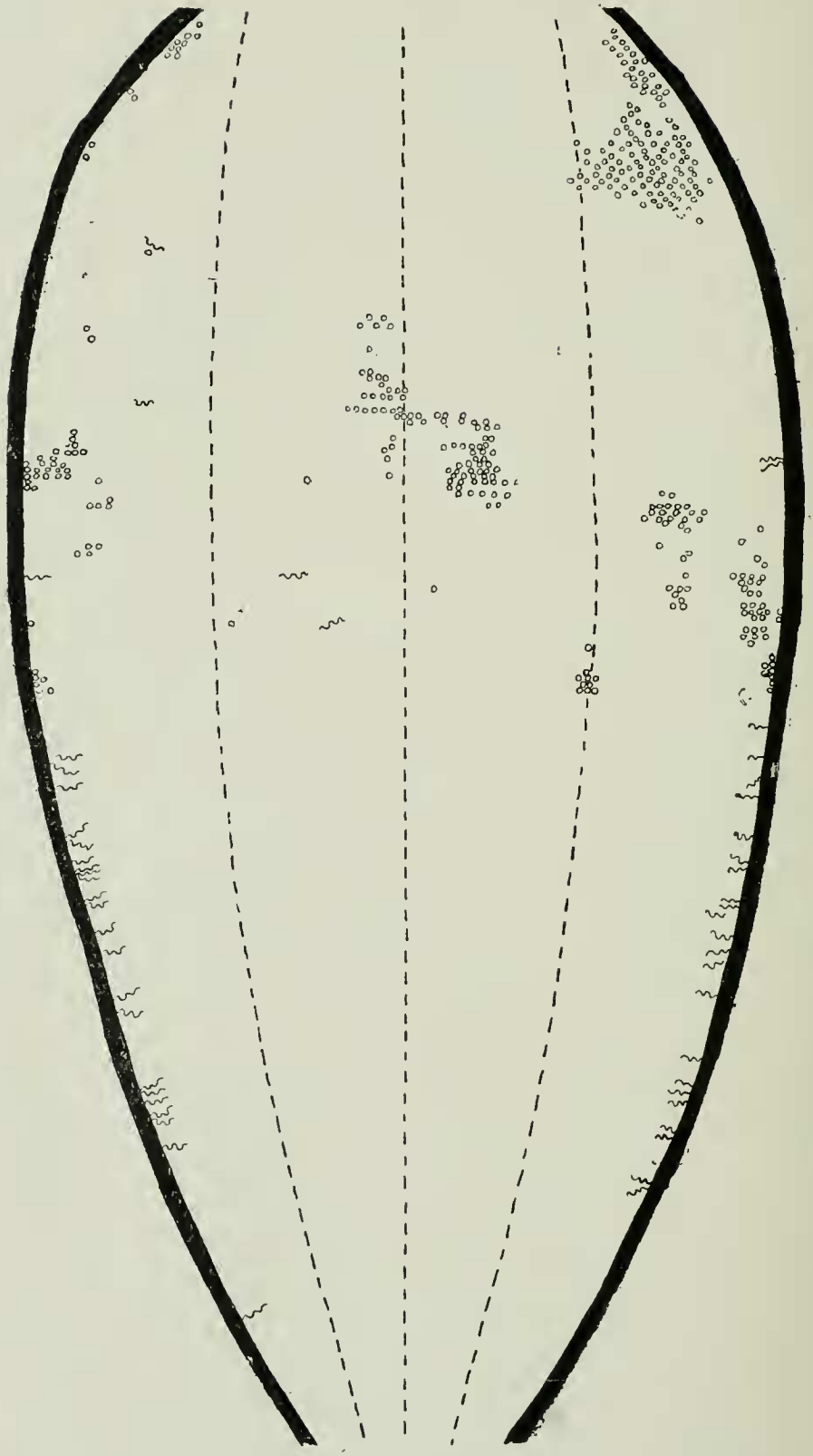
Interpretation.—A stomach which has recently been regenerated and in which the stomach-phase of the trypanosomes is completed and past.

(3) (Text-fig. 5). A large stomach going through about 260 sections. Blood-débris about half absorbed; stains grey. Epithelium for the most part clear, columnar, very granular, with coarse grains and pseudospheres and many yellow bodies. There are a fair number of detached cells, some of them showing yellow necrosis, scattered through the stomach; in the posterior region there are some black degenerated cells still in situ or in process of detachment.

Extracellular trypanosomes very scarce in the anterior half of the stomach; in the posterior half they are more numerous and all attached to the epithelium or peripheral in position.

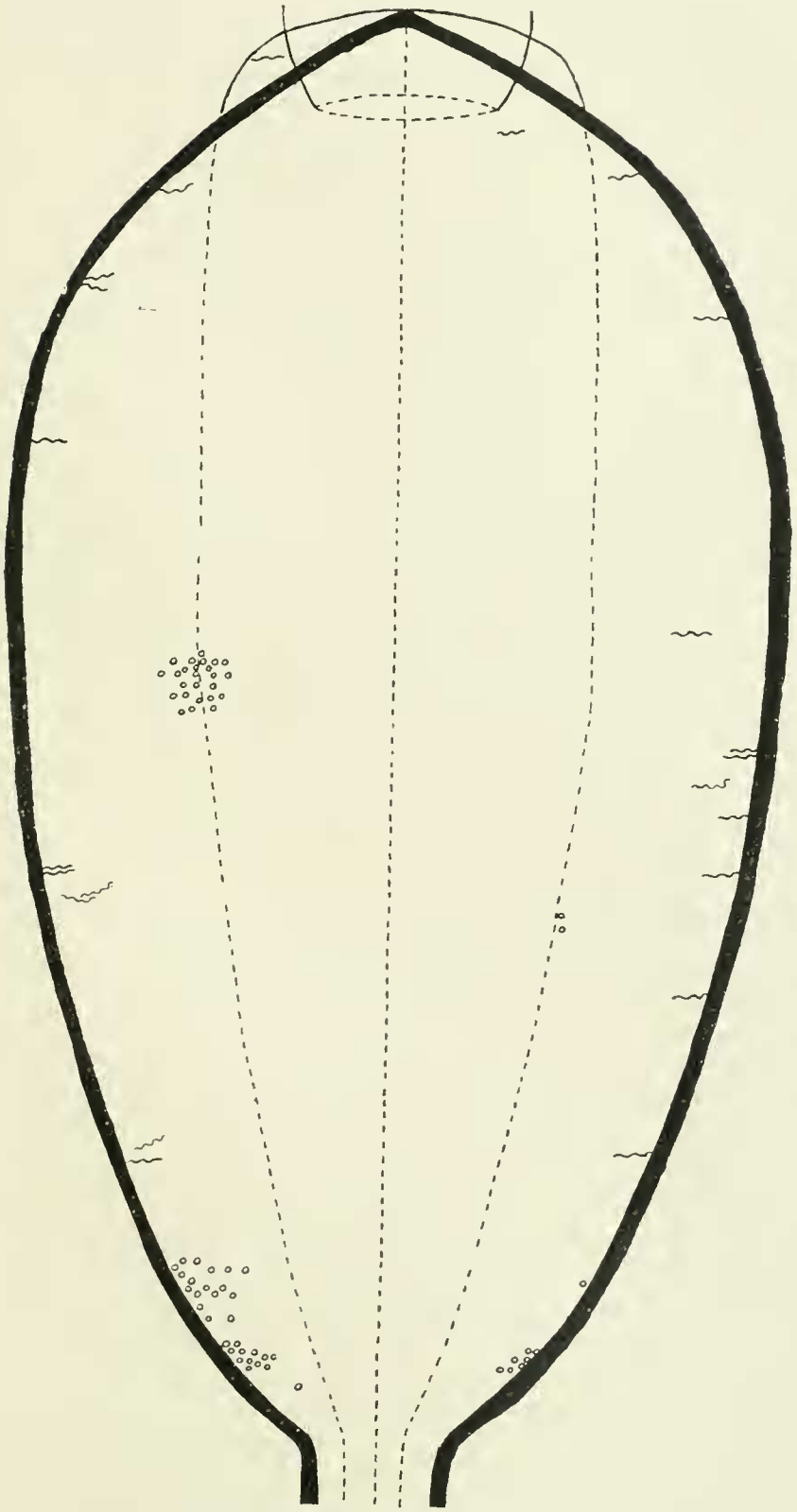
Intracellular trypanosomes are found only in the anterior half, where there are scattered patches, variable in extent, of infected epithelium.

TEXT-FIG. 5.



Reconstruction of another stomach. The series of sections began just behind the proventriculus. 36 hours. Magnified about 111.

TEXT-FIG. 6.



Reconstruction of another stomach. 36 hours. Magnified about 111.

Interpretation.—A stomach very recently regenerated, the process scarcely complete in the posterior region. In the anterior half practically all the trypanosomes have penetrated into the epithelium. In the posterior half an attack is beginning, but all the parasites are still extracellular.

(4) (Text-fig. 6). A large stomach going through 223 sections. Blood-débris as in last. Epithelium as in last, but less granular and with no yellow bodies; no black cells in situ, but a few detached.

Extracellular trypanosomes very few in number, scarcely more than twenty in the whole series; all attached to the epithelium or close to it.

Intracellular trypanosomes in two fairly extensive patches of infected epithelium, one about the middle of the stomach, one close to the pylorus. In one of these patches the cells are badly affected; in the other they are more normal.

Interpretation.—A feebly-infected stomach, recently regenerated, in which almost all the trypanosomes are intracellular.

(5) The stomach runs through 217 sections. Blood-débris large in amount; stained yellow. Epithelium: (*a*) in the first 130 sections mostly very dark, but interspersed with clearer patches of cells; many detached cells, some black, some yellow; (*b*) in the hinder $\frac{5}{8}$, approximately, of the stomach the epithelium is mostly clear, not very granular, with a few patches of black cells in situ, and many detached cells, black or yellow.

Extracellular trypanosomes all confined to the anterior region, in rather scanty numbers for the most part peripheral in position and often attached.

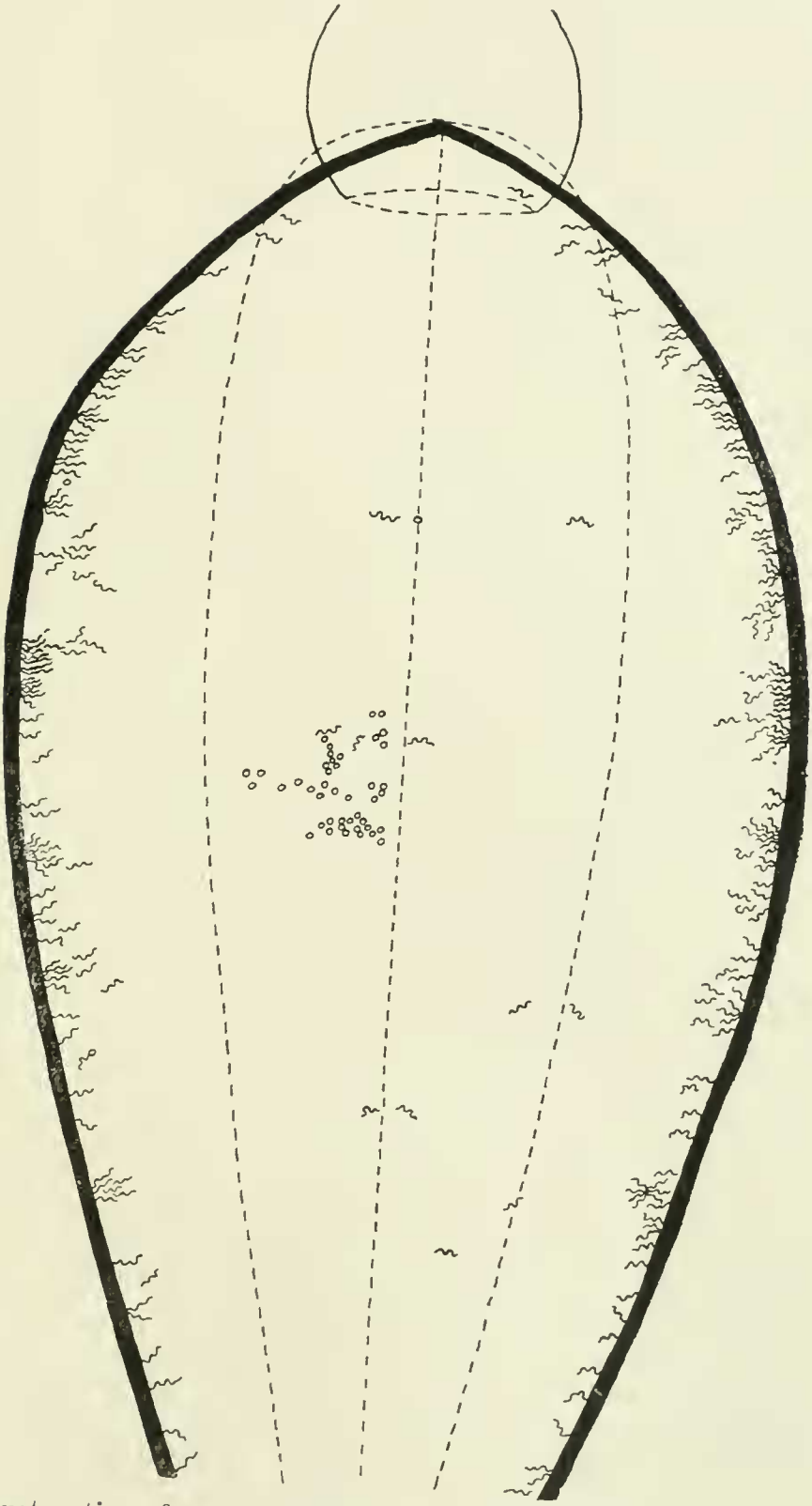
Intracellular trypanosomes found only in the posterior region; three patches of infected epithelium, one fairly large, running through forty-six sections, the cells considerably modified, and two small patches; also a detached cell containing a large sphere in section 132 (rather far forward).

Interpretation.—A stomach in which regeneration is just beginning in the anterior region and is fairly advanced posteriorly. In the anterior region the presence of necrosed cells indicates that there has been a recent attack, but at present there are only extracellular trypanosomes; in the posterior region there are fairly extensive patches of infected epithelium and no extra-cellular trypanosomes.

(6) (Text-fig. 7). The stomach runs through about 240 sections. Blood-débris large in amount; yellow. Epithelium clear, columnar, and with many granules, and in places yellow bodies; interspersed are a few black or yellow cells, singly or in patches, in situ, and detached cells are also fairly numerous.

Extracellular trypanosomes numerous; nearly all close to the epithelium and many attached along the whole length of the stomach.

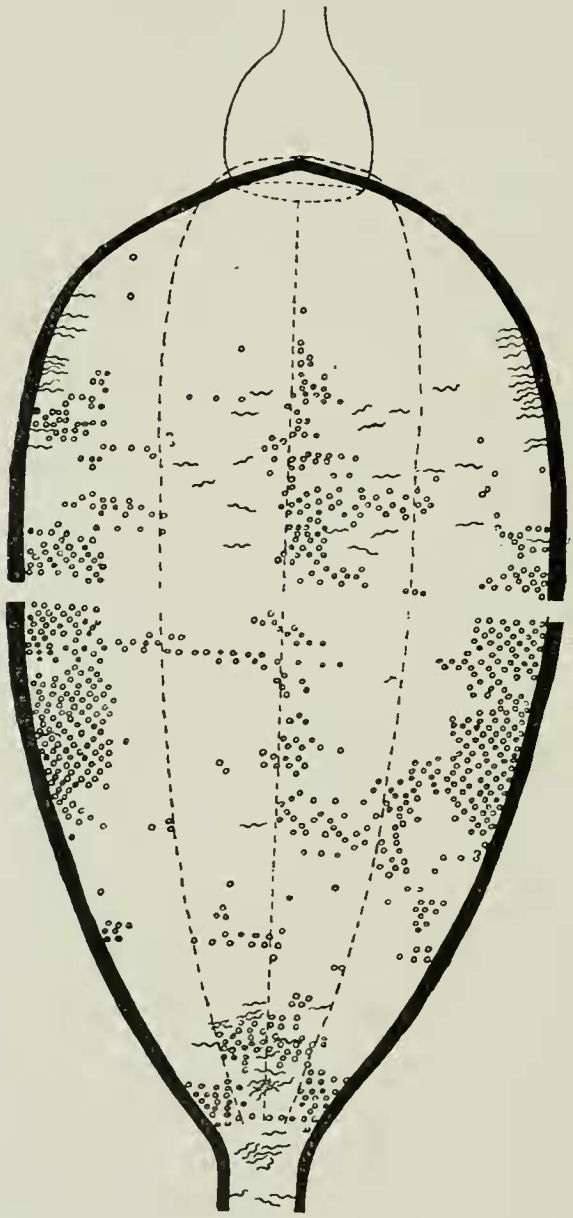
TEXT-FIG. 7.



Reconstruction of another stomach. Owing to the exigencies of space the hinder part of the stomach, in which there were no trypanosomes, has been cut off. 36 hours. Magnified about 111.

Intracellular trypanosomes practically confined to a fairly extensive patch of epithelium about the middle of the stomach.

TEXT-FIG. 8.



Reconstruction of another stomach. The gap in the middle of the figure shows where three sections were accidentally destroyed. 24 hours. Magnified 111.

Interpretation.—A stomach which has undergone regeneration, which is scarcely completed. A previous intracellular generation of trypanosomes, indicated by the presence of necrosed cells, has given rise

to numerous free trypanosomes that are attacking the regenerated epithelium, and have penetrated it and established themselves in the cells in a few places.

(b) Twenty-four hours after feeding.—(7) (Text-fig. 8). From a series preserved in Maier's fluid.[1] A small stomach, running through about 150 sections. In the anterior quarter, or thereabouts, of the stomach are a fair number of attached forms. Behind this region begins a very intense intracellular infection which diminishes somewhat in the posterior fourth, except for an extensive patch close to the pylorus. Through all this region there are practically no attached trypanosomes, but there are a few scattered through the débris, which become more numerous towards the pylorus, and are found passing down the intestine, where some of them are attached to the lining.

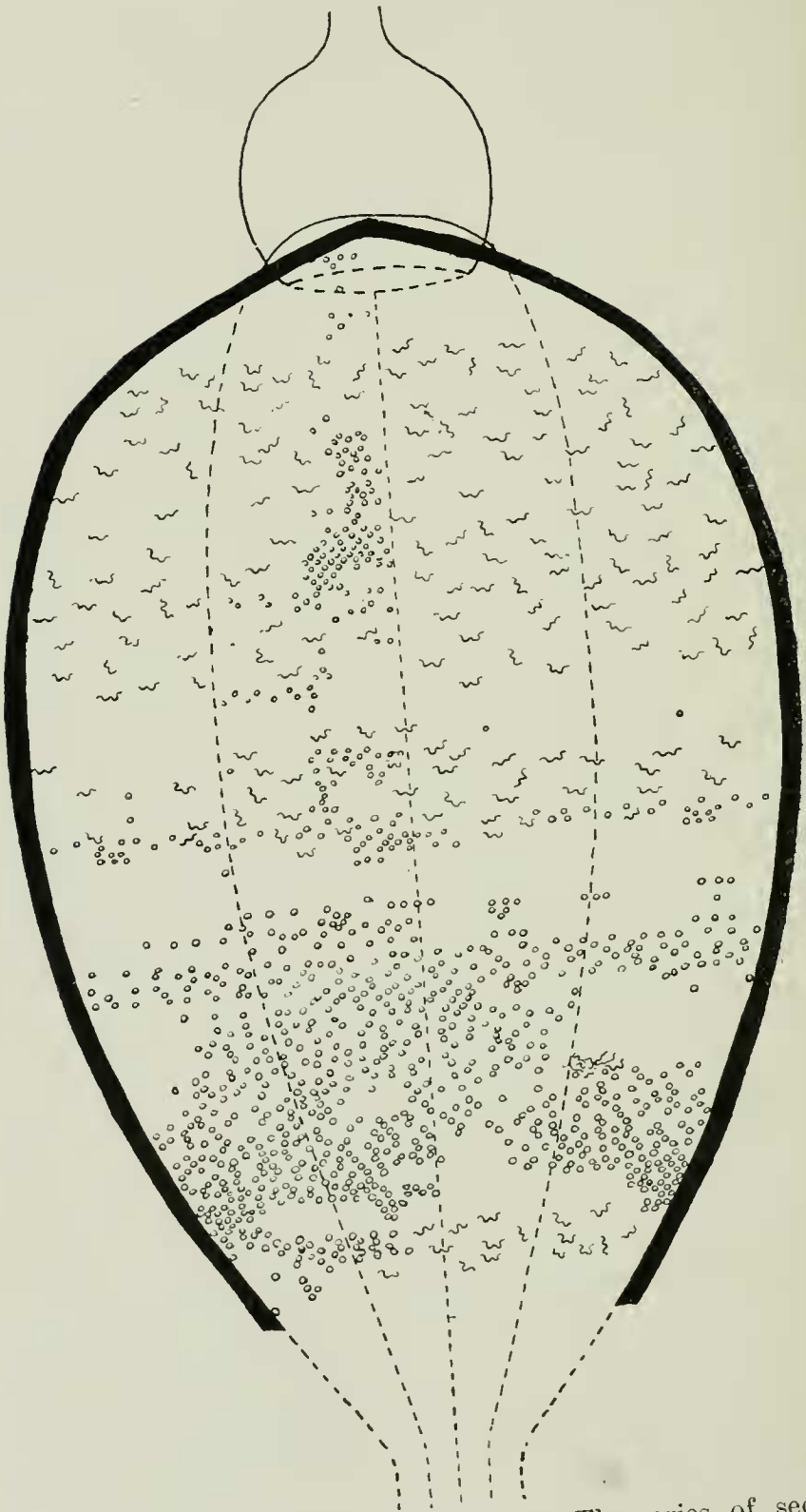
Interpretation.—The intracellular multiplication is at its height and the trypanosomes are beginning to pass on to the rectum, but at the extreme anterior end a fresh attack is developing.

(8) From the same series as the last. A small stomach running through about 154 sections. In the anterior twenty sections the epithelium is columnar, normal, or in places tending to degenerate, with no intracellular trypanosomes, and very few free trypanosomes in the sections. For about forty sections behind this there is an intense infection of the epithelium, which in places is largely destroyed or broken up, and there are great numbers of extracellular trypanosomes, some scattered through the débris, others in close proximity to the stomach-wall. In the next fifty sections, approximately, the epithelium is almost entirely destroyed; only the crypts remain, with here and there a few broken-down cells still adherent to the wall and containing late or completed stages of the multiplication; there are also in this region enormous numbers of extracellular trypanosomes, mostly in close proximity to the wall of the stomach, but also scattered through the débris, in which there are, in addition, masses of detached, broken-down cells, many of them containing large spheres or clumps of trypanosomes. In the twelve sections behind this region the number of trypanosomes and detached cells diminishes rapidly, and in the last thirty-two sections there are no trypanosomes, though still a few detached cells.

Interpretation.—A stomach with an extraordinarily intense infection, which, in the middle region, has destroyed the epithelium completely. The intracellular multiplication has produced vast numbers of trypanosomes, which are not beginning as yet to migrate backwards

[1] In the case of stomachs (7) to (15) the slides of the series were stained alternately with the iron-hæmatoxylin-Lichtgrün-combination and by Giemsa's method.

TEXT-FIG. 9.



Reconstruction of another stomach. The series of sections was not continued quite as far as the pylorus. 24 hours. Magnified about 111.

to the rectum, judging from their absence in the posterior region of the stomach.

(9) From the same series as the last. A large stomach running through about 234 sections. Epithelium almost everywhere columnar, a few flat cells; very few detached.

Extracellular trypanosomes fairly numerous towards the hinder end, mostly peripheral and many attached, a few scattered in the débris. No intracellular typanosomes found.

Interpretation.—A stomach in which one or more generations of intracellular multiplication have been completed and a new attack on the cells is beginning.

(10) (Text-fig. 9). From the same series as the last. A large stomach, but unfortunately not quite enough sections were mounted and the series ends before the pylorus. Blood-débris large in amount everywhere.

In the anterior half of the stomach there are a few extracellular trypanosomes scattered through the blood-débris, very few attached; there are also some intracellular parasites, for the most part scanty, but in one place there is a fairly extensive patch of infected epithelium.

In the posterior half there is a very intense infection of the cells, which in many sections is seen all round the section, or interrupted only by the epithelial crypts. In this region, extracellular trypanosomes are practically absent; one clump, apparently of degenerative forms, was found, and in the hindermost region free trypanosomes, scattered in the débris, begin to appear abundant in the pyloric region.

Interpretation.—Over a large extent of the stomach the intense infection of the epithelium has absorbed, so to speak, all the free trypanosomes except the degenerating forms.

(11) From another series preserved in Maier's fluid. A stomach of moderate size, running through about 180 sections.

Extracellular trypanosomes swarming in the posterior region of the stomach and in the intestine; towards the anterior end they diminish in number, but are to be found right up to the proventriculus. Almost all these trypanosomes are free in the débris, very few are attached.

No intracellular trypanosomes found.

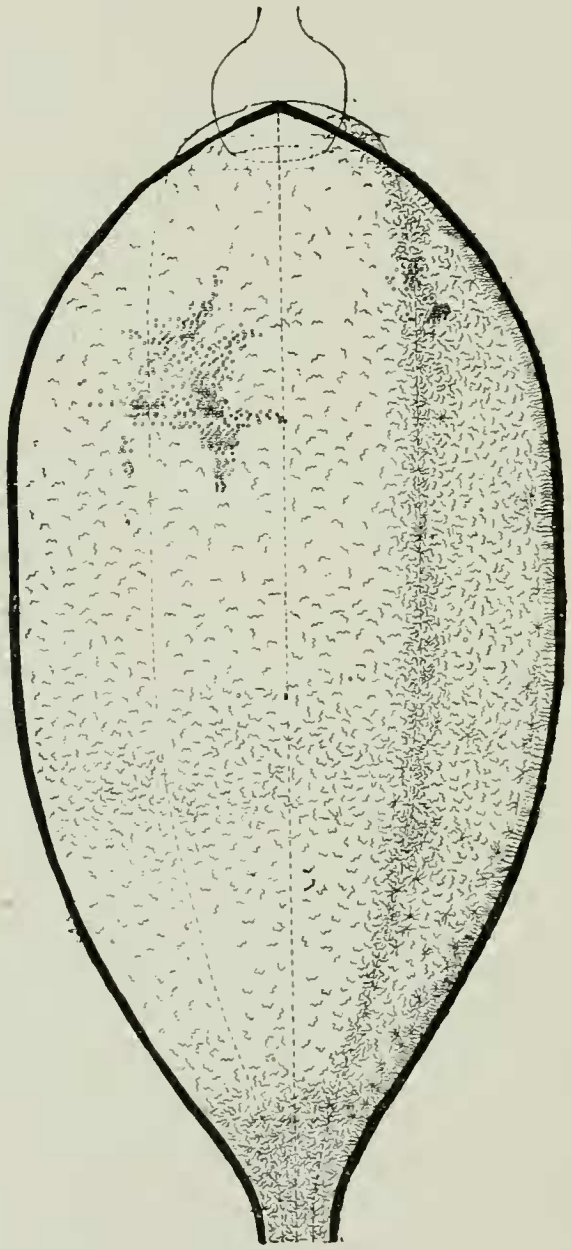
Interpretation.—A stomach in which the intracellular multiplication is practically ended and the trypanosomes, present in large numbers, are passing down to the rectum.

(12) Text-fig. 10). From the same series as the last. A very large stomach, running through about 320 sections, corresponding to a length of nearly 2 mm.

Extracellular trypanosomes occur in vast swarms along the whole

length of the stomach; most of them are peripheral in position occurring all along the east (right-hand) side of the sections, and many

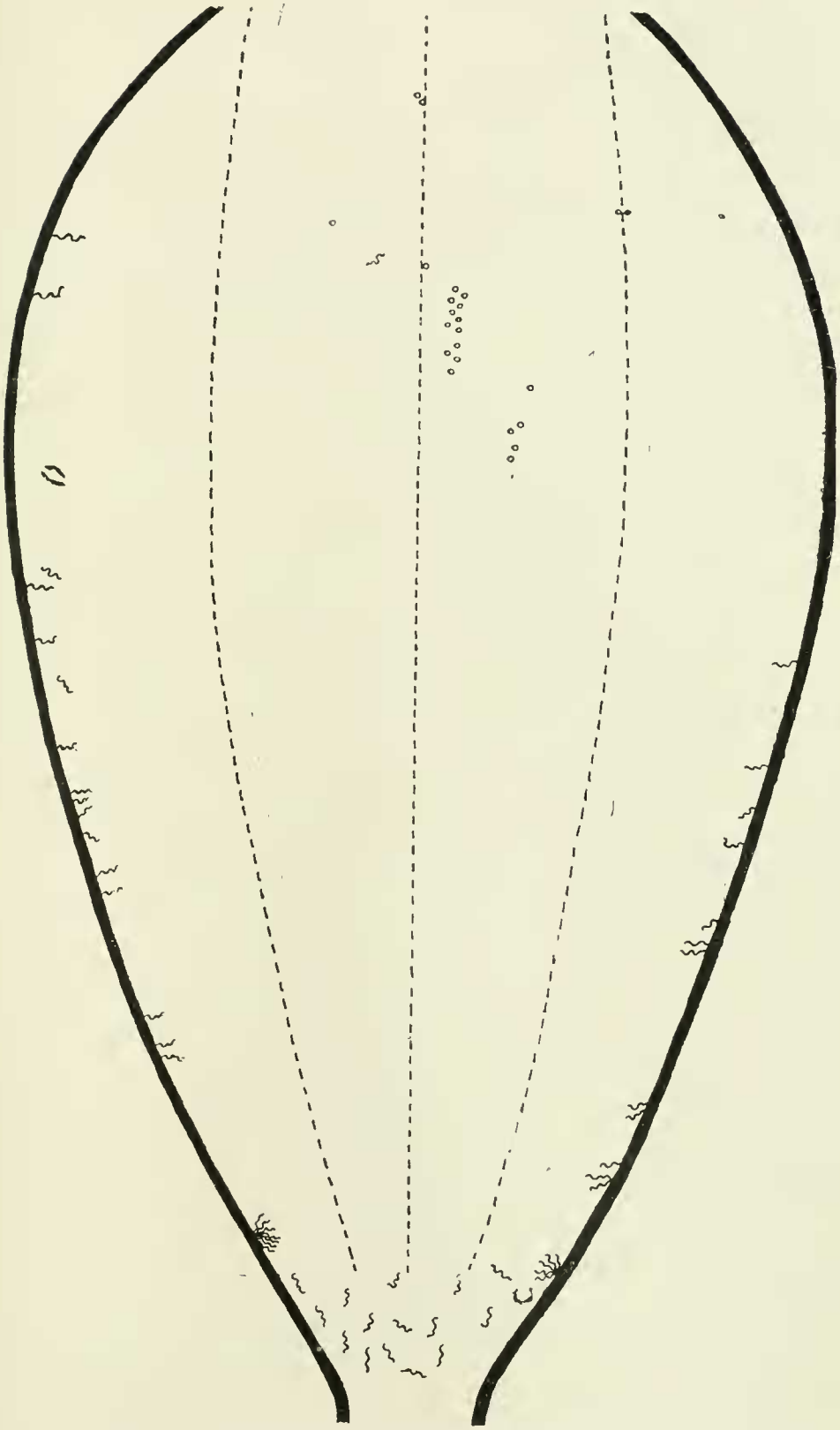
TEXT-FIG. 10.



Reconstruction of a very large stomach. 24 hours. Magnified about 55·5.

of them are attached. Trypanosomes also occur, however, scattered through the débris in all the sections, but more especially in the posterior half of the stomach, and swarms of them are seen in the

TEXT-FIG. 11.



Reconstruction of another stomach. The anterior part of the figure, from just behind the proventriculus, has been cut off owing to exigencies of space, but there were no trypanosomes in the part that has been cut off. 24 hours. Magnified 111.

pyloric region passing down the intestine. Many of these free forms, whether central or peripheral in position, are in clumps and are possibly degenerative.

Intracellular trypanosomes are not found in the posterior half of the stomach, but towards the middle a few detached infected cells are found here and there. In the anterior region there is a very large patch of infected epithelium in situ and also two smaller patches.

Interpretation.— Intracellular multiplication is proceeding actively in the anterior region, and a new attack on the epithelium is preparing all along one side of the stomach. At the same time, trypanosomes are passing down in large numbers towards the rectum and there are many degenerative clumps.

(13) (Text-fig. 11). From another batch preserved in Maier's fluid. A large stomach running through 255 sections.

Extracellular trypanosomes very scanty, practically confined to posterior half of stomach, all attached except in pyloric region, where there are a few scattered freely in the débris. Many of the attached trypanosomes are in clumps, perhaps degenerative.

Intracellular trypanosomes only in anterior region, very scarce.

Interpretation.—The stomach-phase seems to be maintaining itself with difficulty in this flea; the trypanosomes are few in number and largely degenerative, but a few are passing backwards.

(14) Text-fig. 12). From the same batch as the last. A large stomach, running through about 225 sections.

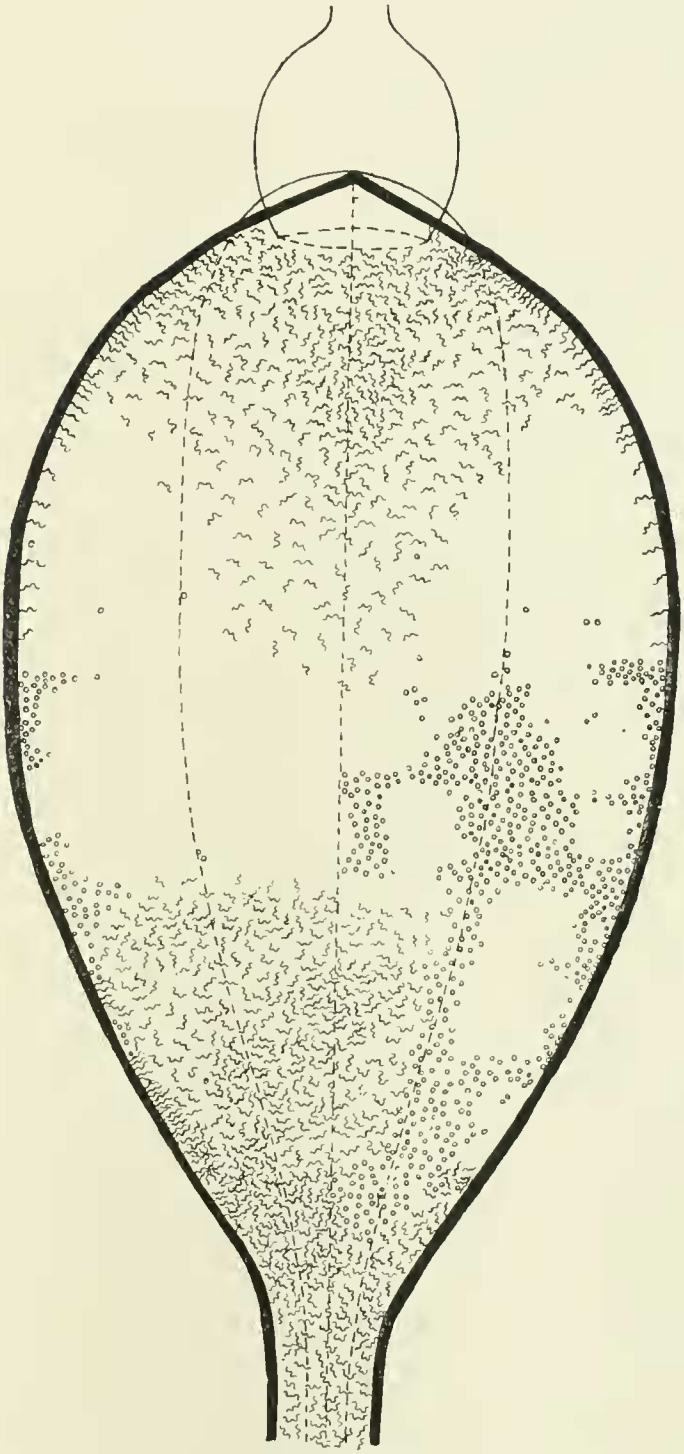
In the anterior third, approximately, of the stomach extracellular trypanosomes are abundant, both free in the débris and attached to the epithelium, but there are no intracellular stages.

In the region behind this, approximately the middle third or fourth of the stomach, there are no extracellular trypanosomes, either free or attached, but a very extensive intracellular infection is found, confined for the most part to the right side of the sections, where almost the whole of the epithelium, with the exception of the crypts, has been attacked; the reconstruction shows very well the clear spaces which represent the situations of the epithelial crypts.

In the posterior third of the stomach a remarkable condition of things is found. On the right side of the sections there are still numerous intracellular trypanosomes, but no attached extracellular parasites; on the left side of the sections there are very numerous extracellular trypanosomes, attached or in close proximity to the epithelium, but none intracellular. Scattered through the débris are many free trypanosomes which are also passing down the intestine.

Interpretation.—A stomach in which intracellular multiplication is still proceeding actively and from which trypanosomes are passing down towards the rectum.

TEXT-FIG. 12.



Reconstruction of another stomach. 24 hours. Magnified about 83·5.

(15) From a batch preserved in sublimate-acetic. A large stomach which passes through 241 sections without quite reaching pylorus.

About 10 extracellular trypanosomes, most of them attached to the epithelium, are found in the hindermost sections.

No intracellular trypanosomes are to be found.

Interpretation.—A stomach in which the intracellular multiplication is either completed or almost inhibited.

(B) The Migration to the Rectum.

As stated above, the trypanosomes occurring in the intestine are usually in transit from the stomach to the rectum, and only exceptionally attach themselves to the intestinal wall or undergo further development there. We have been able on more than one occasion to observe the actual passage of the trypanosomes along the intestine. In a flea which had fed on an infected rat about twenty-four hours previously, the stomach, with a considerable length of the intestine attached, was dissected out on a slide partly teased up and covered with a glass slip; the posterior part of the stomach, however, with the intestine attached, was left intact. Close to the pylorus the stomach contained fluid in which a great number of brown granules were suspended, coarse granules of fæcal appearance evidently representing indigestible residue of the last meal. These granules could be seen to be in a state of violent commotion, more violent than could be explained merely by Brownian movement, since they were being churned and stirred in every direction; but although there could be little doubt but that the movements were due to the activity of trypanosomes, the flagellates themselves could not be seen clearly in the opaque fluid through the stomach-wall. Soon, however, a bolus of fluid passed through the pylorus into the intestine and passed down it by peristaltic action, pushed onwards like a bead, until it reached the cut end of the intestine and was extruded from it. It was then seen at once that the fluid contained, in addition to the fæcal granules, a swarm of excessively active trypanosomes, long and relatively slender forms with great powers of rapid forward progression.

They began at once to spread in all directions in the fluid; but at this moment the coverslip was picked off with needles and dropped instantly into Schaudinn's fluid; after it had been stained and mounted it was found that, by a piece of good luck, the stomach and intestine had remained sticking to the coverslip, and that round the cut end of the intestine were several trypanosomes of the long stomach-type (Pl. 42, figs. 203, 204), evidently some of those that had been seen to pass down the intestine. In two other fleas, fed respectively twenty-four and eighteen hours previously on infected rats, we were able to confirm our observations on the passage of the trypanosomes down the intestine and to obtain preparations of them (Pl. 37, fig. 50).

It is unnecessary to give a detailed description of the migratory trypanosomes, since it is evident from the figures that they are simply of the long stomach-type already described. They are very active, and in form crithidiomorphic, with the hinder part of the body stiff and straight, sometimes slightly or even markedly clubbed and swollen. The two nuclei are more or less closely approximated, but *n* is almost always well behind *N*. It is seen from this that the trypanosomes resulting from the intracellular multiplication in the stomach may do one of two things; they may penetrate again into epithelial cells and go through another generation of multiplication; or they may collect in the pyloric region and be carried down the intestine (compare Pl. 45). The migration may begin as has been seen, as early as eighteen hours, but this appears to be rather exceptionally early, to judge from our observations on the rectal phase; probably it does not usually begin till twenty-four or thirty-six hours. It continues, doubtless, as long as the stomach-phase lasts, and as already stated, we have found intracellular stages as late as five days in fleas not fed a second time; we may suppose, therefore, that the production of the migratory forms and their passage down to the rectum, may be going on continually, in some cases, until the second feed of the flea. In other cases, however, the multiplication in the stomach probably comes to an end of itself,

before the second feed, judging by the many observed instances in which, in fleas not fed a second time, the stomach may contain many long free trypanosomes, or the rectal phase may be well established, without any intracellular multiplication occurring in the stomach.

(c) The Rectal Phase.

The starting point of the rectal phase is the long, active "crithidiomorphic" type of trypanosomes already described, which migrates down the intestine. During its passage down the intestine the changes of form and structure which may have begun already in the stomach continue, so that by the time it reaches the rectum its posterior end is generally distinctly club-shaped. Arrived in the rectum, it very soon undergoes changes in form, habits and structure, and multiplies by binary fission, giving rise ultimately to the typical forms of the established rectal phase, forms which, apart from other characters, are of much smaller size and bulk than those of the stomach-phase. We will discuss first (*a*) the transition from the initial to the established forms of the rectal phase, and then (*b*) the various types of modifications of the latter, culminating in the little trypanosome-form, which is the final stage of the development of the flea.

(*a*) The Transition to the Crithidial Form.

If the various processes of change in the initial stages of the rectal phase be analyzed, after study of both living and preserved material, and by comparing the starting point of this part of the development with its final result, we may note the following tendencies in the organism.

In the first place it loses its intense activity and becomes more sluggish in movement, with a great tendency to attach itself by the tip of the flagellum; under natural conditions the flagellate attaches itself to the wall of the hind-gut, but when under microscopic examination it can be seen to adhere

firmly to pieces of débris of any kind, or to the surface of the glass slide or coverslip. When not attached in this way it progresses slowly with the flagellum directed forwards.

Secondly, the body shortens and changes in form by the cytoplasm becoming concentrated towards the posterior end of the body.

Thirdly, the flagellum becomes progressively shortened.

Fourthly, the two nuclei, if still in their original positions, become transposed into the typical crithidial arrangement, with *n* close beside or in front of *N*.

Fifthly, the nuclei, the flagellum, and finally the whole body are multiplied by division or reduplication.

The order in which these different processes of change have just been stated is in no way to be taken as indicating their chronological order of succession in the development, since they take place more or less independently and, as it were, at different rates of acceleration in different individuals; the result is consequently the production of a great number of forms which at first are rather bewildering and difficult to arrange in a series. The difficulty of tracing in detail the transition from the initial stage of the rectal phase to the established crithidial condition is increased by the fact that the early transitional stages are extraordinarily rare and difficult to find in the permanent preparations; a fact which indicates that the transition takes place very rapidly and is completed very quickly. One explanation of this rapid change may perhaps be found in the great diminution in size which is brought about in this part of the development. Leaving out of consideration for the moment any structural or other changes, it is at least quite clear that the large individuals which come down from the stomach initiate a series of generations of multiplication by fission culminating in forms perhaps not more than a tenth the size of the initial forms from which they are derived. Consequently it is probable that the successive divisions of the body follow one another at first with extreme rapidity and without intervening pauses to allow the daughter-individuals

to grow to the size of the parent, as would happen in the normal multiplication such as takes place in the established crithidial stage. Another explanation for the rarity of the transition-stages may be the possibility that of the long trypanosome-forms which come down from the stomach to the rectum, only a small number may go through their metamorphosis into the typical rectal phase and the greater number may degenerate or be carried out of the flea. Whether this be true or not, it is not necessary to suppose that all those which pass down from the stomach do so in a single swarm; it is more probable that they dribble down from the stomach, so to speak, in larger or smaller bands or singly, a supposition which would also account for the small number found at any one time in the rectum undergoing their metamorphosis.

A further difficulty which may arise in distinguishing the forms of the transition from trypanosome to crithidia, and in assigning them to their proper position in the series, is the fact that the body may become artificially broadened in preparations which have been allowed inadvertently to dry up the least bit before fixation. Deformed specimens of this kind can be recognized by their flattened appearance and consequent even staining of the body, which in a properly preserved specimen should be thicker and more opaque in the axial region than towards the edges of the body; the trophonucleus in flattened specimens is often transversely elongated; and, further, the process of drying seldom affects a single specimen, unless it is very isolated or near the edge of the film, but, if it has taken place at all, the effects of desiccation are apparent over at least a considerable area of the slide or coverslip. It is, therefore, not difficult with a little practice to detect the specimens which have become broadened artificially; and in any case the process of drying does not affect the length of the body or flagellum to any appreciable extent.

It is a result, doubtless, of the rapidity with which the transition is effected that we have not been able to come to a perfect agreement of opinion between ourselves as to certain points of the development during this transitional period,

namely, the exact stage in the process of change of form at which the first division of the initial rectal form (that is to say, the long club-shaped forms that come down from the stomach) takes place, and consequently the type of binary division, whether equal or unequal, which initiates the whole series of generations in the rectal phase. Before we discuss this doubtful point, we may first classify the various types seen in the initial transitional stages of the rectal phase, for which it is simplest to take the types of body-form as the basis of classification. Bearing in mind the considerations of technique that have been discussed already, and being careful, therefore, to eliminate all cases where there is reason to suspect artificial deformation of the specimens, we can recognise the following series of forms, each of which is a stage in the progressive shortening and broadening of the body by concentration of the cytoplasmic substance in the posterior third of the original slender trypanosome :

(a) Slender forms, differing but little from the long stomach type, and evidently but recently arrived in the rectum. The flagellum is still long, and N is usually in front of n (Pl. 42, fig. 208) ; sometimes, however, the reverse is the case (Pl. 42, fig. 209).

(b) Forms in which the hinder region of the body, containing the two nuclei, become swollen and club-shaped (Pl. 42, fig. 206), and the anterior part correspondingly attenuated, until the body as a whole becomes more or less tadpole-shaped (Pl. 41, fig. 150 ; Pl. 42, figs. 205, 207). The flagellum at this stage is usually long, but sometimes distinctly shortened (Pl. 41, fig. 158 ; Pl. 42, fig. 210).[1] The nuclei are usually still in their original relative positions, but are sometimes crithidial in arrangement (Pl. 42, fig. 211).

(c) Forms in which the concentration of the body-substance

[1] How the shortening of the flagellum takes place is not clear, but it is worthy of note that in smears there are often found broken pieces of flagella near the specimens, as if the flagellum had become brittle and had broken off (Pl. 41, figs. 158, 160).

in the posterior half or third of the body has proceeded so far that the body is nearly half as broad as long, and the anterior prolongation which forms the undulating membrane is much reduced in length and very slender. Examples of transitions from the last stage to this are seen in Pl. 41, figs. 151, 152;

Text-fig. 13.

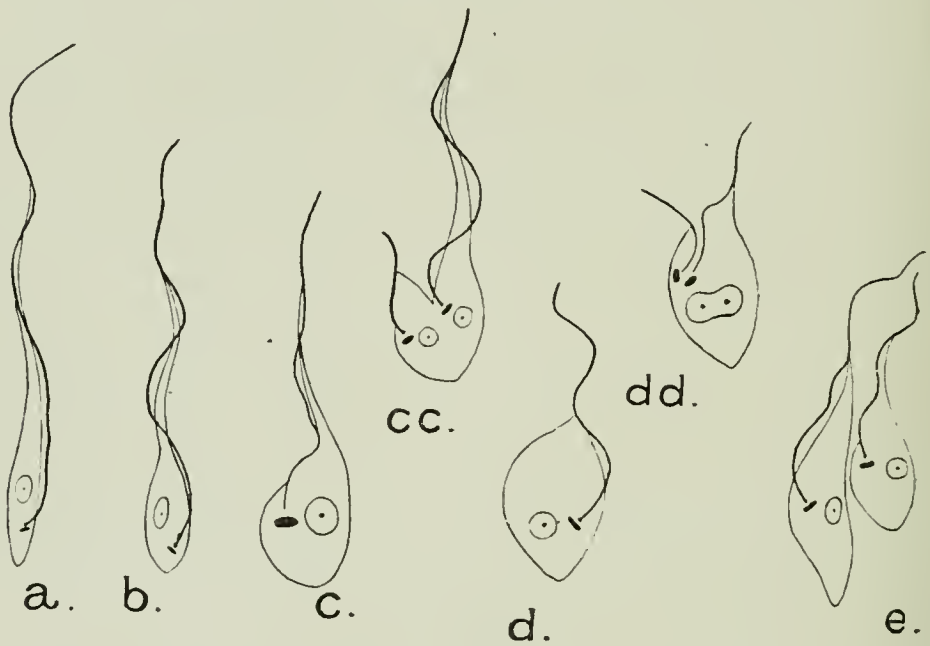


Diagram to show the possible modes of transition from the stomach-phase to the rectal (crithidial) phase: *a*, slender form newly arrived from the stomach (compare Pl. 42, fig. 208, etc.); *b*, early club-shaped form (compare Pl. 42, fig. 205, etc.); *c*, later (more swollen) club-shaped form (compare Pl. 41, fig. 149); *cc*, unequal division of *c* (hypothetical); *d*, large pear-shaped form (compare Pl. 41, figs. 154, 155, etc.); *dd*, division of *d* (compare Pl. 41, fig. 179); *e*, forms resulting from the initial division of the rectal phase (compare Pl. 41, figs. 153, 157, 164, etc.). (× about 2000.)

the complete realisation of this stage is seen in Pl. 41, figs. 149, 154, 162; Pl. 42, fig. 212. From a comparison of the figures it is seen that the position of the nuclei varies considerably; *n* may still be at or near the hinder end (Pl. 41, fig. 162), or may be close beside *N* (Pl. 41, figs. 149, 154; Pl. 42, fig. 212). The hinder end may be pointed, or quite round. The

flagellum may still be a fair length, or quite short (Pl. 41, fig. 162).

(d) Forms in which the body is contracted into a pear-shaped mass, nearly as broad as long. The stalk of the pear is formed by the flagellum together with a slight projection of the body representing the anterior prolongation, now usually reduced to its smallest limits (Pl. 41, figs. 155, 156, 161; Pl. 42, fig. 215). The nuclei are usually transposed, *n* being beside or in front of *N*. The length of the flagellum varies within wide limits represented by Pl. 41, figs. 155 and 156.

(e) Forms which are distinguished from the preceding stage chiefly by their smaller size. Here again, however, caution is necessary in referring a given specimen to its place in the series, since the apparent size in permanent preparations may be affected considerably by technique. In the preparations fixed on the slide with osmic vapour and stained by Giemsa's method the trypanosomes always appear considerably larger than those on the coverslips fixed wet with sublimate solutions, stained with hæmatoxylin and mounted without drying in canada balsam (vide Minchin, 1909). Consequently, different standards of sizes are required for interpreting preparations made by these two different methods of procedure, and preparations made by the one method must not be compared directly, without making due allowance for the differences in result, with those made out by the other method. Nevertheless, after giving due weight to these considerations, it is not difficult to distinguish forms which are about half the bulk of the stages already described (Pl. 41, figs. 153, 159, 160, 164; Pl. 42, fig. 218). Such forms are, without doubt, individuals derived from at least one, possibly more than one, division of the initial form, a conclusion supported by the occurrence of such forms in pairs, possibly as the result of division recently completed (Pl. 41, fig. 157; Pl. 42, fig. 216*a*).

The question which must remain open at present is, in which of the four stages of changes of form (*a*), (*b*), (*c*), or

(*d*) (see Text-fig. 13), does the initial division of the rectal phase take place?

If, as is possible, and as one of us (J. D. T.) thinks probable, the first division takes place in the club-shaped form as soon as the concentration of the protoplasm is completed and when (as in Text-fig. 13, *c*) the two nuclei lie close together, it may well be the case that the products of division would then be markedly unequal in appearance owing to the retention of the old flagellum by one of the two daughter-individuals; that is to say, the division would be of the type found in similar club-shaped forms in the trypanosome of the gold-fish both in cultures and in the leech, and in bird-trypanosomes both in cultures and in the mosquito, and which has also been seen in an early culture of T. lewisi itself. On this view the swollen, pear-shaped forms of stage *d* would have to be interpreted as forms subsequent to, and the products of, the initial division. In spite of much searching, however, through our preparations, we have been unable to find actual examples of club-shaped forms showing division markedly unequal in appearance.

If, on the other hand, as one of us (E. A. M.) believes, it is the most usual state of things for the organism to continue the process of contraction and broadening of the body until it has reached the condition of stage *d*, it is probable that the flagellate would then divide by the type of binary fission characteristic of the subsequent generations of the crithidial phase, that is to say, producing two daughter-individuals that are equal, or not markedly unequal in bulk, and differing only in that one of them has the flagellum temporarily shorter than that of the other. Pl. 41, figs. 179, 180, and Pl. 42, fig. 216, may then be interpreted as examples of the initial division, and Pl. 41, fig. 157 and Pl. 42, fig. 216 *a*, as pairs resulting from the initial division recently completed.

The problem of the initial division of the trypanosomes in the rectum is one which involves more than the question as to the type of form, club-shaped or pear-shaped, in which the division takes place, or the question whether the products of division differ in visible characters of

bulk, structure, or appearance; it raises a much deeper and more fundamental problem, namely, whether the division which initiates the rectal phase is an equating division which gives rise to two equipotential daughter-individuals or a differentiating division which produces inequipotential forms. If the division-products are equipotential, then visible differences between them of any kind would be merely temporary and of no significance for the future behaviour and destiny of the sister-individuals, which would be true twins; all such differences, however pronounced, would be immaterial for the development as a whole, and the same would apply to the parent individual, whether club-shaped or pear-shaped. If, on the other hand, the daughter-individuals are inequipotential, in the sense that the smaller of the two division-products becomes the starting point of an indefinite number of generations of small crithidial individuals, while the larger is merely a "parent" which, though it may divide in the same manner to produce small crithidial forms several times in succession, does not ultimately develop further, but drops out, as it were, of the direct line of the development when its powers of reproduction are exhausted; if they are inequipotential in this sense, it follows that the observed difference between the two daughter-individuals would have an important significance and that they would not then be true twins, and further, that the club-shaped form, assuming that this is the form in which division takes place, would then be a developmental form of special and peculiar significance in the life-cycle and not merely a stage in the change of form leading to the pear-shaped stage. We must be content, unfortunately, with enunciating these possibilities, without being in a position to decide between them; owing to the fact, to which reference has been made above, that the extreme rarity of transitional forms in our preparations has supplied us with insufficient material for a decisive judgment.

The type of the initial division and the exact point at which it occurs in the series of progressive form-changes of the rectal phase must be left at present an open question, unfortunately; but this much may be stated positively about the process of division in the rectal phase in all cases, whether in the initial or subsequent stages. No multiple division occurs henceforth in the developmental cycle, but the parasites settle down to a course of simple binary fission continued indefinitely, and always taking place in the lumen of the gut, never within cells. The process of fission is initiated by division of the blepharoplast or basal granule of the flagellum, but the flagellum itself does not divide; the original or parent

flagellum remains attached to one of the two daughter-blepharoplasts and a new flagellum grows out from the other blepharoplast. The division of *n* follows hard upon that of the blepharoplast, then *N* starts its division, and finally the whole body divides; of the two daughter-flagellates produced, one has the original flagellum, the other has to grow a new one, which it may not do, in some cases, until after complete separation from its twin sister. In any case, one of the two products of division has a much shorter flagellum than the other, irrespective of any difference in bulk between the two.

As already stated, while these changes of form and processes of multiplication are going on the flagellate is also undergoing a change which, though a very small thing in itself, produces nevertheless the characteristic morphological distinction between the trypaniform and crithidial types; namely, the approximation and final transposition of the two nuclei, so that *n* comes to lie beside, or even well in front of, *N*. The approximation of the two nuclei begins with the first alteration and changes of form, but the exact point in the development at which the change of position of the two nuclei is completed is subject to great variation. Very exceptionally, as has been described above, the transposition of the nuclei may be complete in the stomach itself, but more usually, it may be said normally, the change does not take place until the flagellate reaches the rectum. Any of the forms distinguished above as *a*, *b*, *c*, *d* or *e*, may be found with the nuclei, but slightly or completely approximated, and finally in the clumps of developmental forms such as that represented in Pl. 41, figs. 182–184, evidently consisting of forms which have completed several, at least two or three generations of fission since arriving in the rectum, all stages in the process of transposition of *n* or *N* are to be observed. Since the small forms of the established rectal phase show invariably the typical crithidial arrangement of the nuclei, all that can be said is that the change in position of the nuclei is effected earlier or later, but without fail, in the transition from the stomach-phase to the rectal phase.

The successive generations of the transitional rectal phase cannot be distinguished with precision. The size to which the individuals are diminished after a given number of generations is determined by two variable factors during those generations, namely, the rate of individual growth and the frequency with which multiplication takes place. All that can be said is that the rectal forms diminish in size by repeated division until they reach a minimum size which is attained when growth and multiplication balance each other more or less evenly; and that in the rectal infections of recent origin the average size of the individual flagellates is slightly larger, as a rule, than in the old-established infections.

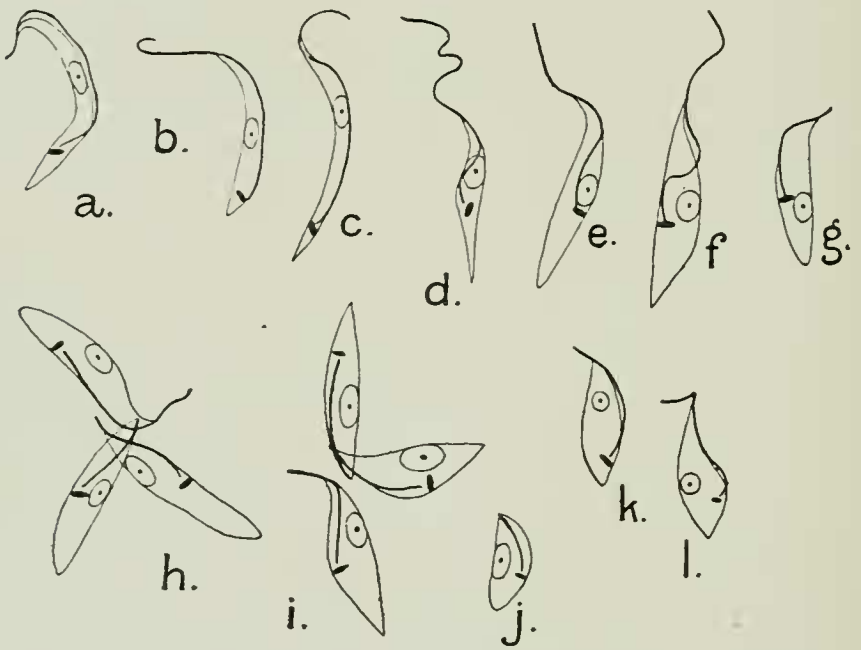
(*b*) The Established Rectal Phase.

In its fully-established and definitive condition the rectal phase consists chiefly of small crithidial individuals which multiply by binary fission, attached to the wall of the gut by their flagella or by the anterior (flagellar) extremity of the body. The crithidial stage appears normally, if not invariably, to take origin in the rectum in the manner described in the preceding section, and this part of the digestive tract is its most usual and characteristic habitat; consequently we have made use of the term "rectal phase" for this part of the development, in spite of the fact, presently to be discussed in more detail, that the crithidial and other forms of this phase may be found not uncommonly in other parts of the gut, more especially in the intestine close behind the pylorus, but also in the stomach itself.

In one flea we have found the rectal phase well-established so early as eighteen hours after the infective feed (Text-fig. 14), and in another flea we found a few typical examples of this phase at twenty-four hours, but both these examples are abnormally early cases of the fully-developed crithidial phase, which, in our experience, is seldom completely established before thirty-six or forty-eight hours after the infective feed.

The extent to which the crithidial infection is developed in different fleas varies greatly, from a swarming infection covering the wall of the rectum like a pile-carpet, especially in the region behind the rectal papillæ, to a condition in which a few scanty flagellates are to be found only by careful searching; in either case, however, the crithidial stage represents

Text-fig. 14.



Various forms from the rectum of a flea eighteen hours after the infective feed, *a*, *b*, *c* and *d* are probably degenerative forms, the rest developmental; *e* and *f*, early rectal forms, *g*–*l*, haptomonads (× 2000).

the permanent stock of the parasites in the flea, multiplying continually and indefinitely and maintaining itself, in all probability, as long as the flea lives. We have succeeded in keeping a single flea alive for nearly three months, and during that time seven rats were infected by it (see p. 640 below). This permanent infectivity can only be explained by the establishment of the crithidial stock in the rectum and its continued multiplication. Each crithidial individual may do one of two things: it may multiply by binary fission to produce two crithidial forms (multiplicative phase); or it may be

transformed gradually into small stumpy trypaniform individuals (final propagative phase). Hence the flagellates of the rectal stock may be classified, broadly-speaking, into crithidial, transitional and trypaniform individuals.

The forms that occur in a well-established rectal infection are very varied, and it is a somewhat difficult task to classify them and to assign to each and every form its due place in the developmental series. To obtain a general notion of the various types met with, it is best to examine fleas some eight or nine days after they have fed once on an infected rat and subsequently on clean rats, so as to obtain the rectal phase in its typical condition, free from admixture of earlier developmental or degenerative forms. Such fleas may have no flagellates at all in their rectum or may present every gradation between an extremely scanty and a swarming infection.

If a rectum containing numerous flagellates be examined fresh after having been dissected out in salt-citrate solution, opened up by tearing it with fine needles, and then covered with a coverslip, the majority of the flagellates are seen attached to the wall of the rectum, chiefly in the region behind the rectal papillæ. Many of them will have become detached as the result of the dissection, and will be seen floating about singly or adhering together in larger or smaller clumps. If the preparation be sealed up carefully and watched for some time many of those that lie about singly will attach themselves to the glass of the slide or coverslip, and it can be seen very clearly in all cases, whether in the forms still attached to the rectal wall or in those set free in clumps or singly, that the attachment is by the tip of the flagellum or by the flagellar extremity of the body in those that have no free flagellum. The flagellates may be attached to the rectal wall in such numbers and so closely crowded together that they resemble a furry lining or pile on it. Seen in profile they appear in serried ranks, each in contact with its neighbours; seen in surface view they present the appearance of a honeycomb, each flagellate in optical transverse section, showing outlines nearly polygonal as the result of mutual pressure. In the living condition the attached

crithidias are motionless for the most part, but occasionally a given individual performs a kind of jerky nutating movement; the body, remaining attached, sways rapidly from one side to the other, with a slight curvature of the axis. After bending, first to one side and then to the other, in this way the animal remains quiescent for a time, but when there are a large number of the flagellates, there is scarcely a moment in which one or another, or several at once here and there, are not performing these movements.

In addition to the attached forms, with the flagellum for the most part very short or wanting altogether, there are usually a certain number of free forms. Some of these are crithidial in form, with the undulating membrane feebly developed and scarcely, if at all, recognisable, and with a distinct free flagellum, often quite long. The function of these flagellated forms appears to be that of migrating in order to colonize other parts of the wall of the gut. When a clump of attached forms has been multiplying actively at one spot it doubtless tends to become overcrowded, at least in its central part. Then probably one of two things may happen; some of the crithidias may become transformed into the final trypanosome-form and detached from the clump; or a certain number will develop flagella, remaining crithidial in type, migrate to another part, and attach themselves again.[1]

Besides the crithidial forms with long flagellum there can be seen free forms, also with a flagellum of variable length, but with a distinct undulating membrane running along the whole or greater part of the length of the body, which appears more flexible and performs sinuous undulatory movements more or less distinctly. These forms are the little trypanosomes, or transitional stages in their development, the forms which constitute the final infective stage of

[1] One of us has seen in the living rectum a crithidia with a long flagellum become detached from the rectal wall, swim actively across the rectal cavity, with its flagellum directed forwards, and attach itself again to the wall on the side opposite to its former attachment.

the cycle in the flea (so-called "metacyclical trypanosomes" of Brumpt).

We can thus distinguish in the rectum of the flea three principal types of individuals, each of which varies considerably in size and details of form, and between which are to be found every possible transition, namely:

(1) The attached or haptomonad[1] form, which is the multiplicative stage of the rectal development.

(2) The free or nectomonad[1] form.

(3) The final trypanosome-form.

The many variations of these three types and the transitions between them cause the rectal phase of the development to present a variety of form which is at first very bewildering, but which becomes easily intelligible if the classification into the three types given above be used as the key. It may be noted here that it is very common, in the development of other trypanosomes, for the crithidial phase to exhibit great variation in size, form, and structure. Compare the works of Chagas on the development of T. cruzi in the bug, and of Miss Robertson (1911, etc.) on the development of the trypanosomes of fishes in leeches.

We will now proceed to describe the variations of these types and the transitions between them in more detail.

(1) The haptomonad or attached type is the phase in which multiplication by binary fission takes place; consequently its variations of form are related mainly to the function of multiplication. Leaving out the forms which are actually in process of division, we find that chief variations in form are seen: first, with regard to the shape of the body, whether relatively slender, with the hinder end sharply or bluntly

[1] Woodcock (1914) has proposed the useful term "haptomonad" for the attached phase of a Crithidia or Leptomonas, the so-called "gregariniform" individuals of Léger, but of which the resemblance to a gregarine is not very striking, not at least in the developmental phases of Trypanosoma lewisi. To denote the locomotor crithidias with long flagella we propose the term "nectomonad," i. e. swimming monad, as a correlative to haptomonad, fixed monad.

pointed, or stouter, with rounded hinder end, or finally ovoid or even globular in form; secondly, with regard to the extent to which the flagellum is developed.

The typical haptomonad, when not preparing for division, has the body spindle-shaped or pear-shaped with the thickest part anteriorly in front of the nuclei, and the hinder end more or less sharply pointed (Pl. 41, figs. 174, 176, 193; Pl. 42, fig. 252). The two nuclei are usually close together, situated either about the middle of the body or nearer to the hinder end; *n* is either just in front of *N* or close beside it. The cytoplasm has a great tendency to stain very dark by any method, especially towards the hinder end. After Giemsa the body has a purplish-blue tinge; after Twort's combination of neutral red and Lichtgrün it is seen to be full of very fine granules, stained red and scattered irregularly (Pl. 38, figs. 260*a*–263*a*); from these reactions it is evident that the opacity of the body is due to the deposition in the cytoplasm of very fine "chromatoid" grains, probably of the nature of volutin. The cytoplasm is usually free from coarse granulations, which are, however, present occasionally.

In preparation for division the hinder end of the body begins to swell and to become rounded at the hinder end, while the nuclei are shifted more posteriorly. In consequence the body becomes pear-shaped, but in the opposite manner to that previously described, since now the thickest part of the pear is the posterior end, while the anterior extremity of the body is narrowed, with the flagellum representing, as it were, the stalk of the pear (Pl. 41, figs. 168, 188; Pl. 42, figs. 231, 247–249). In other cases the body becomes evenly ovoid or even globular in form (Pl. 42, figs. 243–247).

The process of division calls for no special remark (see Pl. 42, figs. 213, 214, 226–230, 249, 253, 266). The blepharoplast or basal granule of the flagellum divides first, one of the daughter-blepharoplasts retaining the old flagellum attached to it, while the root of the new flagellum begins to grow out from the other daughter-blepharoplast (Pl. 42, fig. 252, lowest specimen). Following the blepharoplast, *n* divides

next and after that *N*. Next the body is constricted into two, beginning from the anterior or flagellar end (Pl. 42, fig. 253). We have never seen any but binary fission of the crithidial forms; multiple fission does not occur in any form at this stage. We have also sought without success for multiplication by endogenous budding (the so-called "infective granules" of Balfour and others). In Leptomonas pattoni of the flea we have found very clear instances of apparent endogenous budding (Pl. 42, figs. 281, 282–284), and Pl. 42, figs. 268, 269 are rather suggestive of a similar process occurring in the haptomonads of T. lewisi, but further proof of it is lacking.

The flagellum in haptomonad forms is extremely short, and as a rule is reduced practically to its intra-cellular root or rhizoplast, the projection beyond the limits of the cell-body being very slight or quite imperceptible. At the point where the rhizoplast comes to the surface at the anterior apex of the body there is usually a fairly large and distinct, but often ill-defined patch of substance which stains like the flagellum, that is to say, red, after Giemsa's stain, black or greyish-black after iron-hæmatoxylin, and green with Twort's stain, and which appears to represent a secretion produced by the flagellate, a sort of cement by which the animal adheres to the wall of the rectum (Pl. 41, figs. 174, 178; Pl. 42, figs. 223, 226, 243–245, 260, 262, etc.).

In many cases the rhizoplast fails to reach the surface of the body, or may even, very exceptionally, be absent altogether (Pl. 42, figs. 241–243, 267). The body is then always ovoid or globular in form. In such cases we have true leishmanial forms, which appear to owe their origin to very rapid multiplication of the ordinary haptomonad type; multiplication is so rapid that one of the two daughter-individuals resulting from binary fission has no time to form completely its new flagellum or even the rhizoplast, before being split off from its twin sister and beginning to divide again. This view receives support from the extremely small size which is commonly a feature of these leishmanial forms. In no case have we seen

anything that could be interpreted as encystation or encapsulation of the leishmanial forms; they appear to represent a purely trophic and multiplicative phase.

The haptomonad forms occur usually, as has been said, attached to the cuticle lining the rectum; in surface views of the rectal wall, living or preserved, they may be seen attached singly, in clumps, or in a continuous carpet-like layer, and exactly the same is found in microtome-sections of preserved recta (Pl. 44, fig. 317; Pl. 42, fig. 277). In sections of a well-infected rectum the haptomonad forms are seen in a long, continuous line, like soldiers on parade. But, both in living and preserved recta, in film preparations or in sections, free clumps of haptomonads are also found, in which the individuals all have their flagella directed toward a certain point (Pl. 42, fig. 274). In spite of careful scrutiny it is not possible to detect any body or particle of débris at the centre to which the monads are attached; they appear to adhere simply to one another by their flagellar extremities. The question at once arises whether these free clumps are a natural or an artificial condition. If they were only seen in teased-up recta one could have hesitation in ascribing their detachment to the manipulation, but they are found also in sections of recta. It is, of course, impossible to dissect out and preserve a rectum without subjecting it to great stresses and strains which might detach the monads, but it is remarkable how tenaciously they adhere to the wall. In one of our series of sections of a rectum it can be seen that it has been badly torn in getting it out; part of the torn wall has curled right back and turned inside out. The tear goes right through an attached carpet of crithidias which have nevertheless remained adherent to the wall, even on the part that has curled back, giving at a first glance the erroneous impression that the monads are attached to the exterior of the rectum. They must, therefore, be attached very firmly to the wall, which is intelligible when it is recognised that the crithidial forms are not the ripe, propagative stages of the cycle and that if they were carried to the exterior with the fæces they would be lost. For this

reason alone it seems highly probable that the free clumps of crithidial forms represent either clumps artificially detached by manipulation, or an abnormal condition of the flagellates detrimental to their future welfare.[1]

As transitions between the nectomonad and haptomonad phase we would expect to find both stages of the development of the nectomonad into the haptomonad, and stages of the development in the reverse direction. It is of course almost impossible to say, by simple inspection of a transitional form in a permanent preparation, in which direction it is developing. We are inclined, however, to interpret as transitions from the nectomonad to the haptomonad the more slender forms, with short flagella and hinder ends pointed or but slightly blunted, such as Pl. 41, figs. 175, 176; Pl. 42, figs. 220, 252; and as transitions in the opposite directions the rounded or broad pear-shaped forms with flagella of various lengths, such as Pl. 41, fig. 166, Pl. 42, figs. 231–234, 251, 265. In some forms of the latter type the distal ends of the flagella are very thin, much thinner and more delicate than the proximal portions (Pl. 42, figs. 246, 250, 268, 269), and we interpret this appearance as indicating that the flagellum is in process of growth rather than of regression in length, for the reason that a similar condition is seen in the flagella of forms transitional to the final trypanosome-type (Pl. 42, figs. 255–257), in which the flagellum is not likely to be in process of shortening.

In the series which we interpret as transitional from the haptomonad to the nectomonad type we find globular forms with flagella of considerable length (Pl. 41, fig. 166; Pl. 42, fig. 234), and, since we have not observed such forms swimming freely in the rectum, we conclude that the haptomonad, while attached, first develops its flagellum to a considerable length, and then acquires the elongated form of body, before becoming detached from the wall and set free.

[1] Comparable, for example, to the sponge-larvæ, which attach themselves to the surface-film of the water instead of becoming fixed to a firm object, and which in consequence perish inevitably.—E. A. M.

(2) The nectomonad or free type of crithidial flagellate has a more slender body, in its fully-developed form about five times or more as long as its greatest breadth, but with great variations in its relative proportions (Pl. 41, figs. 190, 194–197; Pl. 42, figs. 217, 235, 236, 254). The body is usually spindle-shaped, pointed at both ends, its thickest part at the level of or slightly behind, the middle point of its length, and *n* is usually well in front of *N*. When it swims the flagellum, directed forwards, is thrown into even sinuous undulations which begin at the tip and run backwards, in contrast to the type of movement so often seen in free-living flagellates, in which the proximal two-thirds, or so, of the flagellum is held stiff and straight, while the distal third performs lashing movements which drag the body forward. We have not found the fully-developed nectomonad type undergoing multiplication by fission, unless Pl. 42, figs. 253 and 266 are to be so interpreted.

(3) The final trypanosome-form appears to rise in most cases from the haptomonad type, with which it is usually found closely associated in preparations; compare Pl. 41, fig. 202, of a section through the intestine close behind the pylorus; the trypanosomes are seen with their posterior ends projecting above the level of the serried ranks of the haptomonad crithidias, as if they were pushed upwards by the development and growth of their flagella. It is possible, however, that the final forms may sometimes arise from the nectomonad type, and that such an origin explains the occurrence of the slender forms of the trypanosomes, the stout forms being derived from the haptomonads. Forms such as Pl. 42, fig. 237, are perhaps to be interpreted as transitional from the nectomonad type to the final trypanosome-form.

The essential feature in the origin of the final form from the crithidial form, of whatever type, is the transposition of the two nuclei, *n*, and *N*. Both nuclei move backwards usually, but *N* only for a short distance, while *n* passes *N* and goes to the posterior extremity of the body (Pl. 42, figs. 238, 239,

255–259, 270). In some cases, especially in the slender forms, *n* stops short of the extreme posterior end of the body (fig. 239), but in the stumpy forms *n* becomes quite terminal in position, as a rule (Pl. 41, figs. 199, 200; Pl. 42, figs. 259, 271). Further characteristic of the final stage is the relatively large size of both *n* and *N*, and the faint stain that *N* usually takes in the permanent preparations. In many cases *N* appears distinctly elongated in the longitudinal direction (figs. 199, 259). With the displacement backwards of *n* and of the attachment and origin of the flagellum, the undulating membrane becomes correspondingly extended and lengthened.

The occurrence of stout and slender forms of the final trypanosomes has been mentioned already, and was pointed out by Swellengrebel and Strickland (1910); but it is a fact somewhat difficult to explain. It may be, as already suggested, that it is simply due to difference of origin, the slender forms arising from the nectomonads, the stout forms from the haptomonads. On the other hand, it may be that the trypanosomes, when ingested by the rat, become exceedingly active in order to find their way from the digestive tract into the blood, and that the slender forms in the flea represent merely the precocious assumption of a type of structure which belongs strictly to a later period of the life-cycle. These are the only suggestions we can offer at present in explanation of the two forms.

We have never in any case seen the final trypanosome-form dividing, but it is stated to do so by Swellengrebel and Strickland (1910), who, after having examined one batch of thirty-seven infected fleas, have been able to figure no less than three examples of a process of division that we have never been able to find in all the many hundreds of infected fleas we have dissected and examined. For our part we agree with Brumpt (1913), that these "metacyclical trypanosomes," as he proposes to call them, are "phases d'attente" which do not multiply further in the flea.

With the development of the final trypanosome-form the

cycle of T. lewisi in the flea is ended. It only remains to say a few words with regard to the occurrence of the rectal phase in regions of the gut situated further forwards than the rectum. It is by no means an infrequent occurrence to find clumps and carpets of various forms characteristic of the rectal phase attached in the intestine and even in the stomach.[1] In the intestine they occur most frequently at the upper end, close behind the pylorus. When they occur in the stomach they are probably always attached towards its hinder end, near the pylorus. Hence the two chief situations of the crithidial forms, when occurring outside the rectum, may be designated briefly "pre-pyloric" and "post-pyloric."

Two possibilities present themselves at once to the mind with reference to these extra-rectal crithidial infections; first, that the infection of the stomach or intestine is a direct one, brought about by forms which have attached themselves there immediately after completing their stomach-phase, without having ever travelled further back in the digestive tract; secondly, that the infection has been brought about in an indirect manner by forms which have migrated forwards from the rectum.

So far as post-pyloric intestinal infections are concerned, we have some evidence that the infection may be sometimes a direct one; in one of our series of sections of the stomach of a flea that had fed thirty-six hours previously to being preserved, there are two large clumps of crithidial forms attached close behind the pylorus. Probably in such cases those attached in the intestine represent but a small numerical proportion of those that migrated backwards from the stomach, the majority having passed on down to the rectum, while a few have stuck, as it were, higher up.

With regard, however, to the pre-pyloric crithidial infections, we have no evidence of direct infection taking place, but all our data indicate that such infections of the stomach are

[1] Since a certain length of intestine was usually cut off with the stomach it is possible that many of the crithidial forms found by us in our stomach-films were really post-pyloric in situation.

brought about indirectly, and the same is probably true, in most cases, of the post-pyloric infections of the intestine. In the first place we have no record of the occurrence of crithidial infections in the stomach (pre-pyloric) earlier than seven days after the first infective feed of the fleas; but at later periods than this we have so many records of such infections in the stomach that, had they been in all cases brought about directly, we should have expected to have found crithidial forms in the stomach during the period when such forms are being established, that is to say, from about thirty-six hours and five days or so, which we have never done. Secondly, the evidence furnished by experiment 39 (see below, p. 634), indicates very strongly that the final infective forms of the life-cycle were first produced in the rectum on the fifth day and were there also on the seventh day, but had migrated forwards to the stomach on the tenth day.

It seems, therefore, most probable that in the majority of cases at least, the pre-pyloric and even the post-pyloric infections are the secondary results of a migration forward from the rectum of crithidial forms previously established there; and since neither the haptomonads nor the final trypanosome-forms appear capable of undertaking such migrations, it must be the nectomonads, which are obviously active locomotor forms, that are responsible for such migration. We have performed some experiments from which it is clear that the migration forwards is dependent on conditions of nutrition in the flea and that starvation favours a forward migration of the nectomonads towards the stomach.

In many cases the forwards migration of the flagellates leads to the rectum being quite deserted by them. This is well shown by the following instance, by no means an isolated one of its kind in our experience, but very typical. A flea was taken from the infected breeding-cage and put by itself on a clean rat for three days, from the 19th to the 22nd of September; it was then recovered and dissected. The stomach-preparations were found to contain a considerable infection of the typical rectal phase (Text-fig. 17, p. 627),

but no flagellates of any kind were found in the rectum. The rat became infected, and first showed trypanosomes in its blood on September 28th. The age of the infection of the flea was not known, but the crithidial stock seems in this case to have died out in the rectum and to have established itself exclusively in the pyloric region.

We have also, though rarely, seen the attached crithidial form in the proximal portions of the Malpighian tubules.

On the other hand we have never seen in our rat-fleas (Ceratophyllus fasciatus) infections such as are described by Nöller (1912), and Wenyon (1913), in the dog-flea, where both the rectum and the intestine are described as being carpeted along their whole extent with the crithidial phase; though we have seen such infections in fleas harbouring the Leptomonas. It is a fact which seems at first strange, but is probably very significant, that, as we have pointed out elsewhere (p. 610), the rat-flea is not so efficient a host for the rat-trypanosome as other species of fleas which do not usually or of choice feed upon rats; from which circumstance it would appear as if the rat-flea has acquired a certain degree of natural immunity to the trypanosome of the rat which other fleas do not possess.

For a general summary of the development of Trypanosoma lewisi in the flea, see Plate 45, and the description of it (p. 691).

(3) The Degenerative Series.

Trypanosomes undergoing degenerative changes may be found in either the stomach or rectum during the first few days after the flea has fed for the first time on an infected rat. They are most abundant in batches of fleas examined during the first twenty-four hours after feeding. After this period trypanosomes may have disappeared altogether from the gut of the flea, and after thirty-six hours degenerative forms are of infrequent occurrence. In some cases, however, degenerative forms may be found in the rectum much later

than the first day, namely, up to three, four, or even five days after the infective feed. The degenerative forms of late occurrence are probably to be interpreted as individuals which have become degenerative after having developed in a normal manner for a longer or shorter period. The trypanosomes which begin to degenerate immediately after being ingested by the flea probably do not last long beyond twenty-four or thirty-six hours, usually not so long. Fleas that have fed on an infected rat whose blood is swarming with trypanosomes often show no trace of the parasites in any part of the gut by twenty-four hours. The majority of the degenerative trypanosomes that are found in the fleas are those that begin to degenerate immediately after being taken up from the rat.

There is no essential difference between the degenerative forms found in the stomach and the rectum. We may, therefore, give a general description of the forms of the degenerative series without taking special note of their provenance.

In direct contrast to the changes undergone by the developmental forms in the stomach, the principal sign of degeneration is a progressive diminution in size, more especially in the length of the body. The trypanosome gradually dwindles and wastes away, beginning at the flagellar end, during which process the flagellum becomes converted progressively into a fluffy mass, which frequently shows a tendency to stain blue or bluish with the Giemsa stain, instead of the normal red (Pl. 43, figs. 294–296, 308). Meanwhile *N* is pushed backwards towards *n*. The displacement of *N* does not appear to be due to any active migration on its part, but to be the purely passive consequence of the dwindling of the anterior part of the body, whereby it is forced backwards. On the other hand *n* shows no tendency whatever to move forwards, but may do one of two things: it may remain where it is, or be shifted backwards only to a slight extent, in which case the hinder end of the body retains the sharp point characteristic of the trypanosome in the blood (Pl. 43, fig. 301); or it may pass back towards the extreme posterior end, and

even become terminal in position, in which case the hinder end becomes bluntly pointed or even rounded (Figs. 290, 291, 308). If at the same time the body becomes broadened out posteriorly, as sometimes happens, the result is a form which may mimic very exactly the small stumpy trypanosome which is the final form of the development (Figs. 299, 306, 307).

The trypanosomes that undergo this process of degeneration show a great tendency to adhere together in clumps attaching themselves to one another by the tips of their flagella (Pl. 43, figs. 304, 308; Pl. 44, fig. 311). The adherence in this way of the degenerative forms must be distinguished clearly from the process of agglomeration which T. lewisi undergoes so readily when placed in unfavourable circumstances.[1] Agglomeration takes place by the hinder ends of the trypanosomes and more especially by their *nn*, as Laveran and Mesnil have shown, and as a result of it the trypanosomes tend to form rosette-like clusters, in which the flagella radiate outwards. True agglomeration of this kind can also occur in the flea under special circumstances, as will be described presently. But in the degenerative clusters the conditions are precisely the opposite to agglomeration, since the flagella are directed towards the centre of the cluster, while the hinder ends of the trypanosomes radiate outwards. The tendency of the degenerative forms to adhere in clumps must be interpreted as an expression of the general tendency (perhaps it might be termed instinct) of the trypanosome to attach itself by the tip of the flagellum to firm surfaces when in the body of the flea, a tendency very pronounced in all developmental forms, excluding the final stage of the cycle.

Clumps and masses of very considerable size are formed by the degenerative forms adhering together in the manner described. Towards the centre the clumps often show a cement-like substance, which stains pinkish-red with Giemsa. The final stages of the degeneration are small forms, which

[1] Manteuffel (1909) has already drawn attention to the distinction between rosettes, with flagella directed inwards, and true agglomeration.

represent simply the hinder ends of the original trypanosomes. They are usually sharply or bluntly pointed (Pl. 43, figs. 302–304), or may be rounded off (fig. 306). The large clumps of these little degenerative forms in the rectum are often very difficult to distinguish in the living state from the clumps of developmental crithidias. The degenerative clumps, however, generally occur loose in the cavity, while the true crithidias are attached to the wall of the rectum, though in the process of dissection the latter often become torn away from the wall. When a loose clump of this kind consists entirely of forms with pointed hinder ends it is probably degenerative. The true crithidial clumps always have a considerable number of forms with rounded hinder ends, especially in the early periods of the establishment of the rectal phase, when the hinder ends of the crithidial forms are almost always rounded. The degenerative forms, carefully examined, show a certain extent of undulating membrane running down the side of the body to *n*, which is situated behind *N*, while in the typical haptomonad phase the flagellum is reduced to the rhizoplast which comes off close to *n* and terminates at the surface of the body, *n* being situated beside or in front of *N*. Finally, it should be noted that the degenerative forms never multiply by division at any time. Nevertheless, in spite of all these distinctions, which are more easily perceived in permanent preparations than in the living state, it is sometimes difficult to pronounce decisively as to the nature of a given individual, whether degenerative or developmental, in preparations of the rectum; but as a rule there is no difficulty at all.

A remarkable fact is the occurrence of recurved forms amongst the degenerative forms in the rectum, of a type essentially similar to the recurved trypanosomes occurring in the normal developmental series in the stomach (Pl. 43, figs. 297, 298). The recurved forms are often seen in the clumps of degenerative trypanosomes. The occurrence of such forms in the rectum may perhaps be interpreted as an abortive effort on the part of the trypanosomes that have

passed on prematurely into the rectum to go through a development similar to that which they undergo normally in the stomach, but which, in all probability, would be impossible in the rectum, where the cuticular lining would doubtless be an effective bar to the penetration of the epithelial cells by the trypanosome. It is possible that some of the recurved forms in the stomach may also degenerate without ever succeeding in penetrating the cells; the curious forms such as Pl. 43, fig. 305, are very probably to be explained as recurved forms in process of degeneration. It would be difficult, however, as a rule, to distinguish between degenerative and developmental trypanosomes in the recurved condition in the stomach; but in the rectum all such recurved forms must be regarded as abortive and destined to degeneration.

As has also been mentioned above, trypanosomes of degenerative type are found in the rectum on the third and fourth days after infection. Such forms may, in some cases, differ but little from ordinary blood-trypanosomes, and are then to be interpreted, probably, as trypanosomes ingested at a later feed, which have passed on to the rectum; but in other cases they may be forms which are undergoing degeneration after having developed normally in the stomach. They are found, not infrequently, mixed with true developmental forms in clumps, into which they have probably intruded themselves (Pl. 41, fig. 183). It is necessary to be careful not to confuse them with early forms of the rectal phase in which n is still behind N; such forms can be distinguished by their greater stoutness and bulk, and by the fact that n has generally migrated forwards to some extent (Pl. 41, figs. 181–187).

True agglomeration very rarely occurs in the flea, but we have found it in its most typical form (Pl. 43, figs. 309, 310) in fleas of a batch, the record of which was as follows: The fleas, twelve in number, had been fed once on an infected rat in the usual way, and three days later they were fed again on a rat, the object being to test the influence of a second feed of clean blood on the persistence of the stomach-phase

(see p. 664 below). By mistake, however, the fleas were fed again on an infected rat instead of a clean rat. The next day (four days after the first infective feed) the fleas were dissected and examined. In every flea the trypanosomes of the second feed could be recognised, quite unaltered from the blood-form, and in most cases agglomerating in pairs, threes, or rosettes composed of many individuals; they were found in the stomach in every flea and in a few in the rectum also, where in one case degenerative forms were noted; in some fleas these trypanosomes were very numerous; in others they were scanty and had evidently undergone reduction in number. The trypanosomes of the first feed had disappeared in nine out of the twelve fleas, while in the remaining three they were represented by developmental forms of the usual type in the rectum. Agglomerating trypanosomes of the second feed were found both in fleas in which those of the first feed had persisted and in fleas in which they had disappeared.

From this observation it would appear that when a flea has once had an infective feed, its digestive tract, and more especially its stomach, acquires properties which cause the agglomeration and probably also the degeneration of trypanosomes taken in at later feeds, alike whether those ingested at the first feed have succeeded in establishing themselves in the flea or not.

Reference has already been made above (pp. 531–532) to intracellular forms which appear to be undergoing degeneration after having penetrated into an epithelial cell (Pl. 36, figs. 43–45).

Appendices to the Development.

(1) Previous Investigations on the Development of Trypanosoma lewisi.

The first who attempted to follow out the development of T. lewisi in its invertebrate host was Prowazek (1905), who studied the development in the rat-louse, Hæmatopinus spinulosus, and since those

who followed immediately after him in similar investigations also made use of the louse, it is simplest to deal first with all those works in which the development in the louse is studied. Since we have not ourselves studied the development of this insect, we are not in a position to controvert the statements made, but it is legitimate for us to compare the forms and stages described with those which we have found in the flea, and, on the ground of such comparisons, to criticise the interpretations given by the authors.

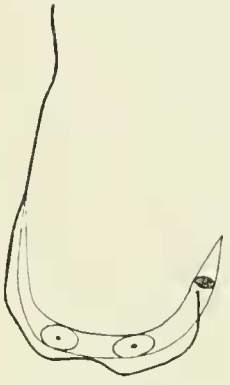
According to Prowazek, the general course of the development in the louse is as follows: The flagellates are first to be found in the stomach, where they do not collect at particular spots, but swim freely everywhere in the ingested blood. In the stomach the processes of maturation and fertilization take place. At the second feed of the louse the parasites are forced down to the end of the mid-gut and finally come to rest in the hind-gut, for the most part near the Malpighian tubules, but also in other parts. Resting stages are to be found on or between the cells of the mid-gut, and more especially at the beginning of the hind-gut. From the fact that the parasites disappear from the hind-gut, it is inferred that they can pass through the epithelium of the hind-gut. [This conclusion is by no means warranted by the observation on which it is founded; it is also, in our opinion, extremely improbable that the flagellates could penetrate through the chitinous cuticle lining the hind-gut.] In this way the parasites are stated to pass into the blood-stream, then into the larynx [sic], and so finally back into the vertebrate host when the louse feeds. [It is not clear whether this statement is founded on observation, or simply on the analogy of the statements made by Schaudinn with regard to Trypanosoma noctuæ; the author never succeeded in obtaining an infection of the rat by means of the louse.] No trypanosomes or their resting stages were found in freshly-deposited fæces.

The author describes in great detail, with figures, various appearances interpreted by him as maturation, fertilisation, and even parthenogenesis. As all this part of the work is in the highest degree unconvincing and appears to consist of forced theoretical interpretations of degenerating forms which were occasionally seen to undergo agglomeration, it is not necessary to do more than refer to the figures given by Prowazek. Pl. II, fig, 32, purports to show the "fertilisation" in the living, and Pl. III, figs. 38 and 39, in the stained condition, while fig. 40 represents the "ookinete," a non-flagellated form with a single nucleus. From the ookinete a crithidial form is stated to arise in the manner described by Schaudinn (Pl. III, figs. 41–43 and 45). An active multiplication follows [but Pl. III, fig. 55, which is given as an example of the division, is simply an unaltered blood-trypanosome which shows the commonly-occurring abnormality of possessing two *NN*; compare our Text-fig. 15].

The author appears to regard most of the crithidias as involution-forms, which form "agglomeration-stars." Such a star is shown in Pl. III fig. 54 [which represents a typical clump of normal developmental crithidias, similar to our Pl. 41, figs. 182–184]. In addition to the crithidial involution-forms there are found other much smaller forms, wedged in between the cells and with the flagella completely absorbed. The crithidial involution-forms may also degenerate into small non-flagellated forms (fig. 50). No special inoculative or final form of the development is described.

Baldrey (1909) professes to have confirmed the development described by Prowazek, including even the process of maturation and fertilisation.

Text-fig. 15.



Trypanosome with two *NN* from the stomach of a flea eighteen hours after the infective feed. Such forms with two and even three *NN* are quite common in the blood of the rat and in the gut of the flea at early stages of the development; compare Minchin (1909), p. 803, Pl. 21, fig. 6, Pl. 22, fig. 74, and Pl. 23, fig. 84; they have nothing to do with reproduction of the trypanosome by fission. (× 2000.)

He gives two text-figures, one showing "male" and "female" forms and "copulation," the other showing "ookinetes" and crithidial forms of the typical nectomonad type. He states that the ookinete reconstructs a flagellar apparatus and divides rapidly to produce crithidial forms, which, by repeated division become smaller and smaller, pass into the body-cavity, thence to the suctorial mouth-apparatus and so infect the rat. The complete cycle takes from eight to ten days.

Rodenwaldt (1909) studied the development of T. lewisi in the louse in order to meet the criticisms of Patton, and succeeded incidentally in proving that Patton's "Crithidia hæmatopini" is a mythical and non-existent species. On the first day of the development he found in the louse both unaltered forms of T. lewisi and long forms,

which he described as "Lanzettformen." [The latter, from the figures given (Pl. 1, figs. 7–12) are clearly the same as our long "crithidiomorphic" stomach-forms.] He also found trypanosomes alleged to be dividing (figs. 5, 6, and 12) [but these, again, are simply forms with two or three *NN*, such as occur frequently in the blood of the rat; see above]. On the second and third days he found the same forms, but a larger proportion of the Lanzettformen. In one louse, however, he found crithidial forms developed on the third day. On the fourth day he found forms with a short flagellum or none at all (as shown in his figs. 13, 14), which he compares with the ookinetes of Prowazek and Baldrey; they are stated to bend their bodies without changing their place. On the fifth day long crithidial forms appear (compare his figs. 15–21), and on the sixth and following days smaller crithidial forms in rosettes, and also, but more rarely, leptomonad forms (figs. 23, 27). From the tenth day there were found (1) small non-flagellated forms (figs. 31–34); (2) stout flagellated forms (figs. 35–39); (3) a few stout, non-flagellated forms, "ookinetes" (figs. 40–47); and also forms regarded as representing copulation of gametes (figs. 48, 49). After twenty days slender, sporozoite-like forms were found (figs. 52–58) in the gut, never in the body-cavity. Rodenwaldt did not succeed in producing infection by means of lice.

Breinl and Hindle (1909) describe the development in the louse mainly as follows. The ingested trypanosomes first show characteristic changes in the nucleus, of which the karyosome divides and the division products move to opposite ends of the nucleus. *N* and *n* then become approximated and a division takes place. Some of the trypanosomes show about this stage a reduction of the cytoplasm, producing tadpole-like forms with a swollen head and the rest of the body reduced to a long flagellum (see their figs. 5–9); at this stage the two nuclei take on the crithidial ("Herpetomonas-like") arrangement. The crithidiæ multiply by division and become "agglomerated in great clusters with the flagellum always directed inwardly" (fig. 18). [These clusters appear to be simply developmental clumps of crithidial forms; as pointed out above, clumps with the flagella directed inwards, whether of degenerative or developmental forms, are not instances of true agglomeration.] Round forms [haptomonads?] are also found (figs. 32–36). The alleged conjugation was not confirmed.

[We cannot help remarking that all the stages of the trypanosome figured by Breinl and Hindle, even the crithidial clumps, present an extraordinarily sickly and degenerative appearance; we venture to think that anyone who compares their figures with ours will agree to this statement.]

Swellengrebel and Strickland (1910), have had the advantage over previous authors that they were able to compare the stages in the louse

with those occurring in the flea; their memoir is illustrated by numerous figures, for which, however, they appear not to claim great exactness, since they refer to them as "diagrams." They find that "the development in the louse is a very irregular one and is not to be compared with that which takes place in the flea." The first changes are that *n* wanders in the direction of *N*, producing finally a crithidial form, slender or club-shaped (diagrams xv and xvi). From the crithidial forms arise large "ovals," some of them without flagella and representing the "ookinetes" of former authors. Later ovals [haptomonads] and crithidiæ [nectomonads] are found singly or in clumps (diagrams xvii and xviii). Degenerative forms were also seen, but no flagellates were found which could be identified with this small, final trypanosome-forms of the development in the flea. No conjugation was observed.

From all these various works, only one positive fact emerges clearly, namely, that T. lewisi can develop in the louse into its typical crithidial phase, with both nectomonads and haptomonads. On the other hand, no intracellular multiplication has been observed, nor has it been proved as yet that the development can proceed so far as to produce the small trypanosomes which end the cycle in the flea. One form, apparently degenerative, occurs in the louse which we have not found in the flea, namely, a large oval form without a flagellum, the zygote or "ookinete" of Prowazek and others.

The first published works on the development of T. lewisi in the flea were those of Swellengrebel and Strickland (1910). We have already noticed above their statements with regard to the development of the stomach-phase and stated that we are quite unable to agree with the account given by them. On the third day of the development they find both long crithidial forms and large "ovals" [stout crithidias of the haptomonad type] in the mid-gut (diagram v). On the fourth day they state that the flagellates had all passed out of the stomach into the intestine. On the fifth day they found only round forms [haptomonads] in the rectum (diagram vii). On subsequent days they found the various forms of the rectal phase, and on the eighth day they found the small trypanosome-forms, the final stage of the development, which the authors were the first to discover. In their diagram xvi, they give a summary of the development showing the following sequence of forms: (1) the normal blood-trypanosomes; (2) a long crithidial form; (3) a stumpy crithidial form with short flagellum; (4) a haptomonad form; (5) the same in process of division; (6) a form transitional to—; (7) a nectomonad; (8) a form transitional to—; (9) the final trypanosome-form. [In view, however, of the great differences seen in the development of the trypanosomes in different fleas, especially prior to the establishment of the rectal phase, it was somewhat rash to attempt to fix the order of events in so few as eighty-three fleas, and it may be

remarked that the entire stomach-phase has practically been omitted from the cycle as summarised by the authors.]

Swingle (1911) gave the following description of the cycle in the flea. He states that the trypanosomes remain but a short time in the stomach, but migrate to the intestine where important changes take place. The first change to be seen is a diminution in size, and at the same time *N* moves towards the posterior end of the body. Occasionally such forms degenerate; in those that do not *n* moves forwards till it is close beside or in front of *N*, thus producing a true crithidial form. The individuals which do not change into the crithidial type curl upon themselves to form an oval rounded mass (figs. 15, 16) [apparently representing recurved forms]. Development of the crithidial forms may proceed along two separate lines which come to the same end; (1) they may "agglutinate" by the anterior ends forming rosettes (figs. 20, 21 [representing typical early crithidial clumps]); or (2) they may form solitary cysts (figs. 22–30) [apparently representing typical examples of the degenerative series]. Other forms [apparently degenerative] are also described; but the haptomonad and other forms of the rectal phase are all referred by the author to the form-series of the leptomonad described by him as Herpetomonas pattoni; a conclusion which Swellengrebel and Strickland (1911–12), justly criticise, though they go too far in the opposite direction in suggesting that H. pattoni is a stage in the development of T. lewisi.

Nöller (1912), studying the development of T. lewisi in the dog-flea (Ctenocephalus canis), confirmed our discovery of the intracellular multiplication in the stomach and added some further details; he observed the penetration of a trypanosome into a cell five hours and fifty-five minutes after the ingested blood had been ingested by the flea and states that the trypanosomes go through at least two generations, probably more, of intracellular multiplication. Whether or not the trypanosomes establish a normal infection in the flea depends, in Nöller's opinion, upon whether they succeed in fixing themselves in the intestine or rectum, or not. As regards the multiplication of the attached forms in the end-gut, Nöller finds that they can always be distinguished from the leptomonads by the possession of a typical undulating membrane and by undergoing a process of multiple fission; neither of these statements accord in the least with our experience of the development of T. lewisi in Ceratophyllus fasciatus.

It remains to mention that Swellengrebel and Strickland (1910) made some observations on the development of T. lewisi in Ornithodoros moubata and Cimex lectularius. In the former they got no development of crithidial forms; in the latter they found large crithidias but no development in the hinder part of the mid-gut.

(2) On the Possibility of the Occurrence of Sexual Phenomena in T. lewisi.

It has been seen from the foregoing summary of previous investigations on the development of T. lewisi that Prowazek first, and after him Baldrey, Rodenwaldt, and Gonder, asserted that the development of T. lewisi in the louse begins with a process of fertilisation, of which the main features are stated to be as follows: Slender male and stout female forms of the trypanosome are differentiated; their nuclei go through a process of maturation and reduction, after which a fusion of the gametes takes place. The zygote is described as an "ookinete" of elongated, oval form, with no flagellar apparatus and with a single nucleus (synkaryon). The nucleus is then stated to divide into two by a heteropolar mitosis to produce the two nuclei of a trypanosome *n* and *N*, and then the locomotor apparatus, flagellum and undulating membrane, are formed. The result is a flagellate of crithidial structure, which proceeds to multiply actively by binary fission.

It must be remarked here that Prowazek's account of the "ookinete" and its development in T. lewisi was modelled in every essential detail on the account given by Schaudinn for Trypanosoma noctuæ. The fertilisation observed by Schaudinn, however, was not that of a trypanosome, but of Hæmoproteus (Halteridium). It is a process of true fertilisation, which was first observed in vitro by Macallum, and its occurrence is not open to doubt. Schaudinn differed from all previous investigators in asserting that the ookinete (zygote) of Hæmoproteus became converted into a crithidial flagellate, a statement which has never been confirmed, and seems never likely to be. There can be little doubt at the present time that in linking together the development of Hæmoproteus noctuæ and of Trypanosoma noctuæ into a single life-cycle Schaudinn fell into error. Prowazek, on the other hand, derived his "ookinete" in T. lewisi from the sexual union and fusion of two trypanosomes, so that in its alleged origin the ookinete of T. lewisi is of quite different nature from that of Hæmoproteus noctuæ. Prowazek is, therefore, the first investigator who claims to have seen sexual conjugation of trypanosomes in the invertebrate host.

Later investigators of the development of T. lewisi in the louse have not confirmed Prowazek's statements as regards the sexual phase, nor has anything similar been found in the flea. Those who have investigated the development of other trypanosomes in their invertebrate hosts have also failed altogether to observe sexual phases or sexual behaviour, in spite of much careful searching for phenomena to which their attention has been strongly directed. As stated above, Prowazek's account of the sexual processes is most unconvincing, and the data he

brings forward are quite inadequate to support the superstructure of theoretical interpretation built upon them. In short, the question of sexuality in trypanosomes may be summed up in the words of Miss Robertson (1912, p. 247): "There is at present no sound evidence of conjugation in any trypanosome life-cycle so far worked out."

We have ourselves searched most carefully, but in vain, for sexual phases and syngamy in the development of T. lewisi. As stated above (p. 519), we found in one flea long crithidial forms adhering in couples in a manner very suggestive of true sexual behaviour, and believed that we had observed true syngamy. We were never able, however, to confirm this observation or carry it any further, and we are now convinced that the phenomena observed on that occasion were simply processes of agglomeration of abnormal forms of the trypanosomes in a malformed flea. When we discovered the stomach-phase we thought it very probable that the sexual processes might take place in this part of the developmental cycle, and we were inclined to interpret as evidence of sexual union some of those stages with two *nn* and two *NN*, such as Pl. 36, figs. 19–23, which are certainly at first sight very suggestive of the fusion of two trypanosomes. We have no evidence, however, of any subsequent fusion of the nuclei, nor of any antecedent processes of nuclear reduction such as should be the preliminary to the process of syngamy. We are not able to arrange the figures of these stages in any series which would suggest a sexual process. In short we are not able to interpret these forms as anything but early stages of the multiplication of the trypanosome.

On the other hand, it has been shown convincingly that the cycle in the invertebrate host effects a marked change in the properties or idiosyncrasies of the trypanosomes that have undergone it. Gonder showed that an arsenic-resistant strain of T. lewisi remained arsenic-resistant so long as it was transmitted from rat to rat by direct inoculation, but lost that property when transmitted by the louse. Miss Robertson (1912) also found that strains of T. gambiense became changed in character when transmitted through the tsetse-fly, and remarks: "It seems clear that the cycle in the fly as a whole, whether conjugation actually occurs or not, has much of the biological significance of the process."

Those who believe that trypanosomes pass through sexual phases in their invertebrate host will be inclined to ascribe the changes in the properties of the parasite to the effects of the sexual process. At the present time it is not possible either to affirm or to deny, with certainty, that sexual processes occur. All that can be said with any approach to verisimilitude is that the change appears to be connected in some way with the metamorphosis of the trypanosome and its passage through a crithidial stage; but proof is lacking that the crithidial stage follows

upon, and is the product of, a sexual process. Attention may be drawn here to another possibility already indicated above, namely, that the erithidial phase may be initiated by a differentiating division into two inequipotential products, one of which is destined to be eliminated sooner or later from the direct line of the life-cycle. If this supposition is correct a possible explanation might be afforded for the renovating effects of the invertebrate cycle. In the present state of knowledge, however, such an explanation must remain hypothetical, and lacking objective foundation.

PART III.—EXPERIMENTAL STUDY OF THE PROBLEMS OF THE TRANSMISSION AND DEVELOPMENT.

(1) Introduction.

Throughout our investigation of the relations of Trypanosoma lewisi to the flea, we have endeavoured, as far as possible, to make experiment and observation go hand in hand, employing the one method to check or throw light upon the results obtained by the other. In the following pages we set forth our results in a number of sections which arrange themselves naturally into two groups. One group (sections i–xv) embraces problems that deal with the complete cycle (including the passage of the propagative forms back into the rat) and with the establishment of T. lewisi in the flea, raising questions that are of general interest in the study of trypanosomiasis. The other group (sections xvi–xix) deals with some further problems that are of interest more especially in relation to the flea Ceratophyllus fasciatus and to T. lewisi itself under more or less special conditions in the flea. Each section is headed by a proposition which it is the object of the experiments cited to establish. If we consider that the proposition is proved satisfactorily by our experiments, it is put in the form of a positive or negative statement; if, on the other hand, the problem stands in need of further proof, the heading of the section is expressed in interrogative form.

The details of each experiment are given when it is cited,

but a few general remarks upon our methods may be made conveniently at this point. We kept going two breeding-cages of the type used by the Plague Commission (see 'Journal of Hygiene,' vi, Pl. iv). In one cage a clean rat was always kept to feed the fleas, in the other an infected rat; these two cages are designated, in the account of our experiments, the non-infected and the infected breeding-cage respectively. From the former we could always obtain a plentiful stock of clean fleas when required, while the latter furnished infective fleas. The rats used were almost always white rats bred in captivity; we found them as a rule docile and good-tempered so long as they were handled with the hands and not with forceps, and the operation of pricking their tails to obtain drops of blood, when required, did not arouse their resentment in the slightest. They live well in captivity, and were none the worse for being exposed to the fleas in the breeding-cages, provided the number of fleas was not allowed to become too great. Many of them suffered, however, from a troublesome itch, caused by a minute Acarine, which is very difficult to get rid of. One of our breeding-cages became over-run by rat-mites, rendering it necessary to destroy it and start a fresh one.

For our actual experiments we used in many cases, especially for experiments with small numbers of fleas, cages of special design in the form of a cylindrical tin-canister with the bottom closed in with tin, the top provided with a lid with a tightly-fitting rim. The canister was 10 in. high and 6 in. in diameter. The top of the lid was made of strong wire gauze, to prevent the rat jumping out, and over that muslin-gauze was pasted to prevent escape of fleas. After these cages had been in use for some time, however, they tended to become rusty on the inside and then the fleas could climb up the tin easily. Consequently, it was found more suitable to use inverted bell-jars, each about 15½ in. in height and 7 in. in diameter. The bell-jars were supported each on a wooden block, or several together in a wooden crate. The open upper end of the bell-jar had a zinc wire cover to

prevent the rat jumping out, but it was not necessary to take precautions against the fleas escaping, because they are unable either to jump so high or to crawl up the smooth glass if kept clean. The bell-jars were cleaned out thoroughly once a week.

The bell-jars were especially suited for experiments with single fleas or a small number of fleas. First of all clean saw-dust is put in the bell-jar to a depth of about 3 in., then the rat and the flea or fleas are put in. Since Ceratophyllus fasciatus is a flea which does not live permanently on the rat but only goes on to it for food, and lives naturally in rat-burrows, the fleas were generally to be found without difficulty in the saw-dust, when it was necessary to recover them, but sometimes they were on the rat itself. In the latter case the rat was held over a deep bowl of enamelled iron and the flea disturbed by blowing on to the fur of the rat, which has the effect of soon making the flea come to the surface of the fur. It was then captured, as a rule, by seizing it gently by finger and thumb, an operation which must be performed rapidly and deftly, otherwise it burrows down into the fur and must be dislodged again. Sometimes the flea drops off the rat and falls into the bowl, where it can be recaptured easily. Our assistant, Mr. George Kauffmann, became exceedingly expert at this job, and if a flea could not be found by him it was safe to assume that it had died or been eaten. In our earlier experiments we used chloroform for recovering the fleas, but later we abandoned this method, often fatal to the rats.

In some cases it was required to expose a large number of fleas—200 or so—to infection on an infected rat for a night or a day. For this purpose the bell-jar was also handy, but it was often found that the rat ate a great many of the fleas, sometimes as many as 100 or more in a single night. To prevent this a cylinder of wire gauze was made, of sufficient length to fit into the bell-jar in such a way that its two ends were closed by the glass wall of the jar, and of such a calibre as to allow the rat to walk forwards or backwards along it,

but not wide enough to permit the rat to turn round or use its paws freely, and consequently hindering it from catching and eating the fleas.

For the purpose of collecting large numbers of fleas from the breeding-cage the following method was found to be the simplest: Two glass capsules were used, each provided with a well-fitting lid, the one smaller, about $2\frac{1}{4}$ in. in diameter and $1\frac{1}{4}$ in. in height; the other larger, about 6 in. in diameter and 3 in. in height. First of all, débris from the breeding-cage containing fleas in all stages of their development is scooped up with the small capsule and the lid at once clapped on. Then the small capsule is placed in the large one; the lid of the small capsule is removed with one hand, and the lid of the large one put on with the other. The adult fleas in the small capsule then begin at once to jump out of it in every direction, and so fall into the enclosing large capsule, in which they soon collect on the side furthest from the light. When the fleas have swarmed out in this way the small capsule is removed and the débris contained in it is returned to the breeding-cage. The fleas in the large capsule can then be emptied through a glass funnel into a suitable receptacle, such as an Erlenmeyer flask. Or, if the large capsule be left to stand until all the fleas have congregated on the side furthest from the light, then by suddenly turning the capsule round through about 180°, so that the side which was furthest from the light is now the most illuminated, the fleas begin at once to move towards the opposite side; and if then the small capsule be held in their way they can be made to jump into it of their own accord, and they can thus very easily be counted and disposed of as required. Ceratophyllus fasciatus is not a very good jumper and its trajectory is low.

The fleas collected can be kept, if required, for a considerable time; we found the best method was to put a little clean white sand, moistened with two or three drops of water, at the bottom of a flask. The fleas burrow down into the sand and appear to live comfortably. If they are to be kept any length of time the sand must be moistened again every

two or three days. Like most blood-suckers, the flea can stand a prolonged fast.

(2) General Problems.

(i) Trypanosoma lewisi is transmitted from Rat to Rat by the Rat-flea, Ceratophyllus fasciatus.

It is not necessary that we should cite experiments specially to prove this proposition, since it is established by the experiments brought forward under the headings that follow, and it has been proved beyond all possibility of reasonable doubt by experiments already published by others as well as by ourselves.

The agency of fleas in the transmission of T. lewisi was first demonstrated by Rabinowitsch and Kempner (1899), who succeeded in infecting clean rats by intra-peritoneal injection of teased-up fleas (species not stated) which had previously been fed on infected rats. In these experiments the trypanosomes appeared in the blood of the rats in six to eight days after the injection. The authors state that they were not able to find any stages of the trypanosome in the flea-débris which was injected. They also obtained positive results by placing fleas, previously fed on infected rats, upon clean rats; the trypanosomes made their appearance in the blood of the rats after two to three weeks. Their experiments with lice gave negative results.

In spite of the experiments of Rabinowitsch and Kempner, the work of Prowazek (1905) on the development of T. lewisi in the rat-louse, Hæmatopinus spinulosus, led to this insect being regarded as the true host of the rat-trypanosome, and no more experiments with fleas appear to have been undertaken until those of Nuttall (1908), who obtained positive infections of rats with fleas, using both Ceratophyllus fasciatus and Ctenophthalmus agyrtes. Two years later we published accounts of a number of experiments, since when the rôle of the flea has been established beyond the necessity of further experiment upon the subject.

We have confined our experiments throughout solely to the common English rat-flea, Ceratophyllus fasciatus, but it has been shown by Nöller (1912) and Wenyon (1913) that the transmission can be effected by other species of fleas, namely, the dog-flea, Ctenocephalus canis; the human flea, Pulex irritans; and the Indian rat-flea, Xenopsylla cheopis. It is indeed noteworthy that other species of fleas appear to be more efficient as true hosts of the rat-trypanosome than the species which in this country occurs habitually

in association with rats, since Dr. Wenyon has informed us that in the fleas with which he experimented, the trypanosomes never failed to establish themselves and to go through their complete developmental cycle, while in Ceratophyllus fasciatus we found that only a small percentage of the fleas became infective (see below), and examination of the fleas showed that the trypanosomes establish themselves in a correspondingly small percentage (see p. 659). It would appear, therefore, that the flea which, more than any other species, is exposed in this country to infection by T. lewisi, has developed a certain degree of natural immunity to the parasite. From the experiments published by the authors cited it is probable that T. lewisi would undergo its development in any species of flea, and would be transmitted by it, provided that the flea could be induced to suck the blood of an infected rat. The natural efficacy of any given species of flea in transmitting T. lewisi depends probably on the habits and tastes of the flea, and not on any specific ability to harbour the trypanosome. Brumpt (1913) has pointed out that all the trypanosomes of small rodents seem to be able to develop in fleas.

A number of experiments have been performed by several investigators on the transmission of T. lewisi by means of the rat-louse, Hæmatopinus spinulosus. The first experiment with rat-lice (species not stated) was carried out by MacNeal (1904), who transferred "several" lice from an infected to a clean rat; trypanosomes appeared in the latter after fourteen days. Positive results in experiments of this kind with rat-lice are reported by Nuttall (1908), Baldrey (1909), Breinl and Hindle (1909), Manteuffel (1909), and Gonder (1911). To judge, however, from the published accounts of these transmission-experiments, positive results were by no means frequent and were obtained in some cases at least with difficulty and by the exercise of great patience and perseverance, or by using large numbers of lice. Nuttall obtained one positive result in an experiment in which sixty lice were used; two other experiments, in which fewer lice were used, were negative. Baldrey reports two experiments in which infection was obtained by means of lice; in the first, 100 lice were used, and the result is regarded by him as a case of direct mechanical infection, but for what reason is not at all clear; the second, in which ten lice were used, is interpreted as demonstrating a developmental cycle in the louse. Breinl and Hindle report three successful transmissions by means of lice, after carrying on numerous experiments for over a year. Manteuffel seems to have been more successful than most other experimenters in this field, though he does not record the actual number of his experiments or the proportion of those which were positive in result, but he states that infections with lice were "prompt and frequent"; his method was to put infected rats, with lice on them, in the same cage with clean

rats, and from his results he concludes that lice do not transmit the trypanosome longer than from three to five days after being removed from the infected rat, and that the transmission is effected by the act of blood-sucking; if the first of these two conclusions be true, it would appear that the trypanosome does not succeed in establishing itself in the louse in the way it does in the flea. Gonder reports that after many fruitless attempts to transmit T. lewisi with definite numbers (80–100) of lice, he obtained six positive results in a series of fifty experiments, and eight positive results in another series of fifty, using greater numbers (grössere Mengen) of lice; and he also brought about six infections by making emulsions of lice taken directly from an infected rat, the lice having been left on the infected rat for five, nine, eleven, thirteen, sixteen, and twenty-one days respectively, in these six experiments. On the other hand, Prowazek, who first described developmental stages in the louse and claimed that this insect was the true host of T. lewisi, was unable to obtain experimental transmission; Rodenwaldt obtained no positive results with numerous transmission-experiments; and we also have obtained only negative results in any attempts that we have made to transmit T. lewisi by means of the rat-louse.

It is evident from the results summarised briefly in the foregoing paragraph that transmission of T. lewisi can be effected by the rat-louse, but only with difficulty, and in a small percentage of cases. This is a great contrast to the ease and comparative certainty with which the trypanosome can be transmitted by fleas. We have always used our flea-cages as the simplest and easiest method of obtaining infected rats when required by ourselves or by our colleagues or friends, and not only have we infected rats with single fleas on many occasions, but we have even succeeded in infecting several rats successively with one and the same flea. We have no hesitation, therefore, in regarding fleas as the usual agency whereby T. lewisi is transmitted from rat to rat in Nature, a result brought about by the louse rarely and exceptionally.

It should be noted that Brumpt (1913) has succeeded in infecting a rat with T. lewisi by inoculating it with the rectal contents of a bug, Cimex lectularius, fed on an infected rat thirty-eight and again six days previously. There is no evidence, however, that this insect transmits the infection naturally.

(ii) The Transmission takes place by the Cyclical Method. Transmission by the Direct Method has not been proved to occur.

These are among the conclusions drawn from experiments described in full detail in our preliminary report (1910). It

is sufficient here to state that experiments "A" (20) and "B" (21) in our report were devised chiefly to separate "direct" from "cyclical" infection, a matter of primary importance at the time that these investigations were begun, and they show that in the individual cases cited (A_3 and B_2) transmission was effected when all possibility of the direct method was excluded. Experiments "C" (22) and "D" (23) multiply such cases many times, and show further that fleas once infective retain the infection so as to infect a series of rats without themselves being exposed to fresh infection. Since then a number of experiments have been carried out by us, many of which are enumerated under the different headings which follow, and the sum-total of these experiments not only supports the conclusions in our preliminary report, but establishes beyond doubt that the rat-flea is a true intermediate host, that it can transmit the infection to other clean rats only after the developmental cycle has been completed within itself; that, in short, the infection takes place by the cyclical method; and that there is no evidence whatever to show that the rat-flea is capable of carrying the infection from one rat to another by what is called the "direct" or "mechanical" method.

The term method in the phrase "method of transmission," if used without qualification, should include comprehensively all that happens in the transmission of infection from one vertebrate to another. In the transmission of trypanosomes the natural transmitting agent, when such is known, is a blood-sucking invertebrate of some kind. When the method is said to be "contaminative" or "inoculative," transmission is viewed from the side of the invertebrate in its relation to the vertebrate, and the problems involved are particular, that is to say such as deal with modifications due to special circumstances in those relationships, and are concerned at most with special groups of trypanosomiases rather than with trypanosomiasis in general. On the other hand when the method of transmission is said to be "cyclical" or "direct," transmission is viewed from the side of the trypanosome in its relation to the invertebrate, and the problem becomes a general one, dealing with that phase of the transmission which is concerned with the life-history of trypanosomes as a group of parasites, and with the wider question of their double relationship to vertebrate and invertebrate,

bringing them into line with other known relationships among parasitic Protozoa in this respect.

A great impetus was given to the study of trypanosomes by economic and other considerations arising out of the prevalence of tsetse-fly disease and sleeping sickness in Africa. It was long known that these diseases could be transferred artificially by direct inoculation of blood from a diseased to a healthy subject by means of a hypodermic syringe. Naturally, therefore, before much work had been done in this direction tsetse-flies known to be associated with the spread of these diseases were supposed to transmit them in this direct way. Bruce and others, experimenting with bred-out flies, proved the possibility of this taking place under certain conditions which, in the case of sleeping sickness at all events, were very unlikely ever to be fulfilled in Nature. Only with a swarming infection and by interrupted feeding could the disease be passed on directly from an infected to a clean animal with any approach to certainty, and even under the most favourable conditions in other respects, the longer the interval between interrupting the feed on the infected animal and continuing it on a clean animal, the less the chance of the clean animal becoming infected, until, with the lapse of about half-an-hour, it was just as certain that the infection would not take place. Moreover, however short the interruption between the feeds, an interposed partial feed on a clean animal rendered the fly non-infective to a second clean animal. Later experiments showed that the contents of the stomachs of flies that had fed on an infected animal, if injected into a clean animal, could produce infection only up to about two days after the infective feed. The fly itself, however, could not be shown to act in any way resembling a hypodermic syringe, and the idea of "delayed mechanical transmission" never found support from feeding experiments. The conclusion to be drawn from all the earlier experiments on direct transmission seemed to be that when infection was obtained it was with "fouled proboscis" before the blood in its lumen distal to the entrance of the salivary duct, and perhaps also on its external surface, had had time to dry, and that the conditions under which it was shown to be possible were never likely to be fulfilled in Nature, in the case of sleeping sickness, and in the case of tsetse-fly disease of cattle, far too seldom to account for the spread of the disease, while in no case could such a method of transmission account for the existence of fly-belts through which healthy domestic stock cannot pass. Other things being equal, the efficiency of an invertebrate as a transmitter of trypanosomes would be enormously increased if the invertebrate were a true intermediate host and not merely a "porter" of the parasites from an infected to a clean subject, and to demonstrate beyond doubt that trypanosomes underwent an alternation of

generations was of primary importance in connection with the general trypanosome problem at the time that we undertook this investigation, when it was being maintained by Patton and others that no trypanosomes went through a developmental cycle in the invertebrate, that all transmission of trypanosomes was direct, and that the crithidial forms found in blood-sucking invertebrates were all of them independent parasites of the invertebrate, having no connection with the trypanosomes or other parasites of the vertebrate. It was known that rat-fleas could transmit T. lewisi from infected to clean rats, and although transmission by fouled proboscides seemed quite out of the question, it was necessary to demonstrate beyond doubt that the rat-flea is a true intermediate host of T. lewisi, that it can transmit the infection to other rats only after the developmental cycle has been completed within itself, and that once infected it remains infective for a considerable time, so as to be able to infect a series of clean rats without itself being exposed again to infection. These points, which we believe concern the transmission of trypanosomes in general, and which may be taken as typical of the relations which trypanosomes as a group bear to their invertebrate hosts, as well as other points of more special interest (confined, it may be, to T. lewisi alone or to the lewisi group), are dealt with under different headings in what follows later. Although the practical cannot properly be separated from the scientific, the most interesting problem of the transmission from the scientific point of view is perhaps the way in which the trypanosome becomes established. There are considerable variations in the details of the cycles of different species or groups of trypanosomes in their natural hosts due to special conditions, but arising out of the very meaning of a cycle, and therefore common to all is the fact that until the cycle is completed the invertebrate, though infected, is not infective. This may be of direct practical importance in special cases, and where that is so it is important to ascertain the length of time required for the completion of the cycle in each case. Of more general practical importance in questions connected with the spread of infection is the fact that the trypanosome does establish itself in such a way that the invertebrate remains infective for a long time without requiring to be exposed again to infection.

(iii) The Trypanosomes make their Appearance in the Blood of the Rat Five to Seven Days after Infection; the Multiplication of the Trypanosomes in the Blood of the Rat come to an End Eleven to Thirteen Days after Infection.

In order to establish with exactness the length of the

incubation-period and the multiplication-period in the rat after infection, it is necessary that the rat should have been exposed to infection by the fleas for a short time; long exposure leaves too wide a margin between the possible maximum and minimum deducible from the actual data furnished by the experiment for the mean to be of any value in reckoning the length of the two periods in question. When the rat is removed from contact with the infected fleas it is further very necessary that all fleas should be removed from its skin. The rat is then kept in a flea-proof cage and its blood is examined daily in fresh, living films until trypanosomes are first detected in it, in order to determine the duration of the incubation-period; then smears of the blood are made and preserved daily and examined until the multiplication-period is found to be past and ended. So long as the trypanosomes are multiplying in the rat's blood, they are of various sizes, some of the ordinary, normal size, others very small, and others again much above the normal size. Marked variation in the size of the trypanosomes is a sure sign that multiplication is proceeding, even when actual division-stages are so scarce in the preparation that prolonged search is necessary in order to find them. As soon as the multiplication is ended the trypanosomes are all of one type and size, allowing for slight individual variations that are not perceptible without careful measurement; to such trypanosomes, the normal form of T. lewisi and the sole form occurring in the blood when once the multiplication is at end, we shall refer always as "ordinary."

We cite here a few examples from our series of experiments, choosing first (Table C), those in which the rats were exposed to infection for one day only, so that the periods of incubation and multiplication can be determined within a margin of one day. In our second table (D), we quote those instances in which the rats were exposed to infection for two days, so that a wider margin of possible error must be allowed for in calculating the two periods. In a third table (E), we shall give some results obtained with rats which were infected

TABLE C.—One Day's Exposure to Infection.

No. of experiment.	No. of rat.	Put in.	Taken out.	Trypanosomes first seen.	Multiplication ended.	Incubation period.	Multiplication period.
20 (= A)	122	7 : xii : '09	8 : xii : '09	14 : xii : '09	18 : xii : '09	6–7 days	10–11 days
21 (= B)	123	7 : xii : '09	8 : xii : '09	14 : xii : '09	18 : xii : '09	6–7 ,,	10–11 ,,
22 (= C)	129	10 : xii : '09	11 : xii : '09	16 : xii : '09	23 : xii : '09	5–6 ,,	12–13 ,,
23 (= D)	138	16 : xii : '09	17 : xii : '09	21 : xii : '09	Rat died	4–5 ,,	—
40	317	15 : v : '11	16 : v : '11	21 : v : '11	26 : v : '11	5–6 ,,	10–11 days
45	371	28 : vii : '13	29 : vii : '13	3 : viii : '13	9 : viii : '13	5–6 ,,	11–12 ,,

TABLE D.—Two Days' Exposure to Infection.

No. of experiment.	No. of rat.	Put in.	Taken out.	Trypanosomes first seen.	Multiplication ended.	Incubation period.	Multiplication period.
20 (= A)	130	11 : xii : '09	13 : xii : '09	17 : xii : '09	22 : xii : '09	4–6 days	9–11 days
22 (= C)	121	6 : xii : '09	8 : xii : '09	13 : xii : '09	19 : xii : '09	5–7 ,,	11–13 ,,
,,	124	8 : xii : '09	10 : xii : '09	14 : xii : '09	21 : xii : '09	4–6 ,,	11–13 ,,
,,	132	11 : xii : '09	13 : xii : '09	17 : xii : '09	24 : xii : '09	4–6 ,,	11–13 ,,
,,	135	13 : xii : '09	15 : xii : '09	20 : xii : '09	26 : xii : '09	5–7 ,,	11–13 ,,
,,	141	20 : xii : '09	22 : xii : '09	28 : xii : '09	1 : i : '10	6–8 ,,	10–12 ,,
23 (= D)	164	13 : i : '10	15 : i : '10	21 : i : '10	26 : i : '10	6–8 ,,	11–13 ,,
,,	168	18 : i : '10	20 : i : '10	27 : i : '10	30 : i : '10	7–9 ,,	10–12 ,,
24	153	6 : i : '10	8 : i : '10	14 : i : '10	18 : i : '10	6–8 ,,	10–12 ,,
,,	156	8 : i : '10	10 : i : '10	15 : i : '10	21 : i : '10	5–7 ,,	11–13 ,,

TABLE E.—Rats Infected by a Single Flea.

No. of rat.	Flea put on rat.	Flea recovered.	Trypanosomes first seen.	Multiplication ended.	Incubation period.	Multiplication period.
207*	2 : viii : '10	5 : viii : '10	11 : viii : '10	13 : viii : '10	6– 9 days	8–11 days
212*	19 : ix : '10	22 : ix : '10	28 : ix : '10	30 : ix : '10	6– 9 ,,	8–11 ,,
223*	10 : x : '10	14 : x : '10	17 : x : '10	22 : x : '10	3– 7 ,,	8–12 ,,
233	20 : x : '10	25 : x : '10	28 : x : '10	31 : x : '10	3– 8 ,,	6–11 ,,
244	11 : xi : '10	15 : xi : '10	23 : xi : '10	26 : xi : '10	8–12 ,,	11–15 ,,
253*	27 : iii : '11	1 : iv : '11	5 : iv : '11	7 : iv : '11	4– 9 ,,	6–11 ,,
259*	23 : iii : '11	27 : iii : '11	30 : iii : '11	4 : iv : '11	3– 7 ,,	8–12 ,,
263*	20 : iv : '11	25 : iv : '11	1 : v : '11	5 : v : '11	6–11 ,,	10–15 ,,
265*	14 : ii : '11	18 : ii : '11	24 : ii : '11	27 : ii : '11	6–10 ,,	9–13 ,,
272*	14 : ii : '11	18 : ii : '11	24 : ii : '11	28 : ii : '11	6–10 ,,	10–14 ,,
291*	27 : iii : '11	1 : iv : '11	5 : iv : '11	9 : iv : '11	4– 9 ,,	8–13 ,,
292*	25 : iv : '11	29 : iv : '11	1 : v : '11	6 : v : '11	2– 6 ,,	7–11 ,,
296*	15 : iv : '11	20 : iv : '11	22 . iv : '11	25 : iv : '11	2– 7 ,,	5–10 ,,
295*	17 : iii : '11	22 : iii : '11	27 : iii : '11	31 : iii : '11	5–10 ,,	9–14 ,,
315*	29 : viii : '11	left on	12 : ix : '11	16 : ix : '11	14 days (max.)	18 days (max.)
361*	13 : vii : '12	,,	23 : vii : '12	26 : vii : '12	10 ,, ,,	13 ,, ,,
363*	13 : vii : '12	,,	23 : vii : '12	26 : vii : '12	10 ,, ,,	13 ,, ,,

* The rats marked thus were examined only on alternate days. Consequently the dates of the appearance of trypanosomes in their blood, or of the termination of the multiplication period, may have been a day earlier than stated in the table.

TABLE F.—Rats Infected by Injection of Fleas.

Experiment.	No. of rat.	Date of injection.	No. of fleas.*	Trypanosomes first seen.	Multiplication ended.	Incubation period.	Multiplication period.
39	309	29 : iv : '11	10 (r)	8 : v : '11	10 : v : '11	9 days	11 days
39	312	1 : v : '11	10 (s)	8 : v : '11	13 : v : '11	7 ,,	12 ,,
39	314	4 : v : '11	10 (s)	10 : v : '11	15 : v : '11	6 ,,	11 ,,
39	257	15 : xii : '10	6 (s)	21 : ii : '10	26 : ii : '10	6 ,,	11 ,,
34	261	26 : i : '11	6 (s)	1 : ii : '11	6 : ii : '11	6 ,,	11 ,,
34	282	14 : ii : '11	1 (s)	20 : ii : '11	(not noted)	6 ,,	—
32	287	14 : ii : '11	1 (s)	20 : ii : '11	,,	6 ,,	—

* r = rectum; s = stomach.

each by a single flea, in order to show that in many cases, at least, the maximum periods of incubation and multiplication which can be deduced from experiments under these conditions are not greater than those indicated by the experiments in which many fleas were used to obtain infection. In a fourth table (F) we give for comparison, the results obtained by inoculating rats with the stomachs or recta of fleas; in such cases the length of the periods of incubation and multiplication can be determined with exactness, the moment of infection being known.

The determination of the length of the multiplication-period in an infected rat is of practical importance for interpreting other experiments, since, when it has been determined, it furnishes a datum from which approximately accurate conclusions can be drawn as to the time at which the rats become infected, when the point is shown definitely by the details of the experiment. As regards the first appearance of the trypanosomes in the blood, they appear at first in such scanty numbers that it is very easy to overlook them, and they may often be reported absent when a more prolonged search would have detected their presence. Similarly, the trypanosomes at the end of the multiplication-period may sometimes have been reported as "all ordinary" in a smear in which more careful searching might have led to the discovery of a few individuals above or below the normal size. Consequently, the errors of observation are such as tend to over-estimate the length of the incubation-period, and to under-estimate that of the multiplication-period, from the scrutiny of the blood-films. On the whole, however, the results obtained in our experiments are very uniform and indicate an incubation-period of about six days, a multiplication-period of about twelve days. It is interesting to note that these results agree with those obtained in the case of rats infected artificially by inoculation, intra-peritoneal or otherwise, with blood from an infected rat. Since a syringe would inoculate far more trypanosomes than the rat would obtain from even a large number of fleas, it might have been expected that the rat would, so to speak, fill up quicker when infected by means of a syringe, and that consequently the multiplication-period would be correspondingly shorter. In our experience, however, the length of the multiplication-period remains approximately constant in all cases, whether the infection is effected by a syringe, by a large number of fleas, by a few fleas, or even by a single flea; a fact which indicates that the length of time during which the trypanosome multiplies in the rat

is not determined by the number of trypanosomes put into the rat, but by the mutual interaction of host and parasite.

It may be noted here that some rats appear to possess a certain degree of natural immunity to infection with T. lewisi. A single instance which came under our experience will suffice to demonstrate this point. A rat was exposed to infection on June 9th and its blood was examined daily; on June 25th a few trypanosomes were first seen in the blood in scanty numbers, just as they are usually seen at their first appearance between the fifth and seventh days of the infection. The rat was then removed from contact with the fleas and kept apart; but neither on the next day nor on any subsequent day were any trypanosomes to be found in its blood. This rat. therefore, contracted only a transitory infection which was late in its appearance and disappeared after one day; had the trypanosomes been overlooked on that day the experiment would have been returned wrongly as negative in result.

(iv) The Cycle of Development in the Flea requires a Minimum of Five Days for its Completion.

This point was dealt with in our preliminary communication (1910), in which we came to the conclusion that the incubation in the flea was six or seven days. Our method of determining this was, first of all to expose non-infected fleas to infection, by putting them on a well-infected rat, for but a single day, so that if the fleas afterwards produced an infection, the time at which they themselves became infected could be determined within a narrow margin, twenty-two hours in our actual experiment. The fleas were then placed in contact for three days with clean rat (1) which did not become infected; after that for three days with clean rat (2), which also did not become infected; and then for two days on clean rat (3), which showed trypanosomes in its blood six days after being removed from contact with the infected fleas. Clean rat (3) was, therefore, infected by the fleas in the interval between the sixth and eighth day after the fleas themselves had acquired the infection; consequently the infection in the fleas could not have been more than eight days old.

Subsequent experiments performed by us have indicated a possible minimum of five days for the flea-cycle of the try-

panosome. In experiment 39 (see below, p. 630) it is proved that the rectum of the flea, injected into the rat, can produce an infection as early as the fifth day, and in such fleas the examination of films shows the presence of the small trypanosomes which are the final form of the development in the flea. In experiments 26 and 28, undertaken in order to ascertain whether a rat, in which the trypanosomes are still in the multiplication-period, is capable of infecting fleas (see below, p. 657), the results obtained indicated a short incubation-period in the fleas. Thus in experiment 26, 127 fleas, after being three days (from 8 : ii : '10 to 11 : ii : '10) on the infected rat were put on rat 187 for another three days (from 11 : ii to 14 : ii). Rat 187 showed trypanosomes in its blood after five days (19 : ii), and the multiplication-period ended five days later (24 : ii). Consequently the incubation-period in the fleas could not have been more than six days (8 : ii to 14 : ii). In experiment 28, 137 fleas were put first on the infected rat for four days (15 : ix : '10 to 19 : ix : '10), and then were put for one day (19 : ix to 20 : ix) on rat 209; after this they were put on rat 215 and left on it. Rat 209 had shown no infection when it died nine days later (29 : ix); rat 215 first showed trypanosomes on 30 : ix, and the multiplication was ended 3 : x. This result indicates that rat 215 was infected about 21 : ix, in which case the incubation-period in the flea could not have been more than six days (15 : ix to 21 : ix). Since rat 209 showed no trypanosomes for at least nine days after being exposed to infection it was probably not infected; so that the infection in the fleas was probably not ripe for at least five days (15 : ix to 20 : ix).

On the other hand we have instances, as already mentioned in our preliminary communication (1910), of an incubation-period in the flea apparently much longer than six days. Thus in experiment 19 a cage (J) was stocked with seventy-two fleas from the non-infected breeding-cage and an infected rat (No. 81, a wild black rat, naturally infected) was put with them for three days (20 : ix : '09 to 23 : ix : '09). Rat 81 was then removed and rat 82, a clean, tame rat, was put in its place (23 : ix) and left in the cage. Rat 82 first showed trypanosomes in its blood 29 : x; the multiplication-period was ended about 4 : xi, indi-

cating that the actual infection of rat 82 took place about 23 : x. In this case, therefore, the fleas did not produce an infection in the clean rat for at least a calendar month after their contact with the infected rat was interrupted. Such a result, however, permits of no conclusion whatever as to the length of the incubation-period in the flea; it merely demonstrates a point proved also by other experiments, namely that infective fleas often fail to infect. We have put forward already (1910) one possible explanation for this, that a rat, which is comparatively immune to begin with, may resist infection for a long time, but its resistance may be overcome at last. Another possible explanation may be given by the method in which infection of the rat by the flea is now known to take place, namely by the rat licking off the moist fæces of infective fleas that are deposited on its skin (see below, p. 648). It is evident that if the rat fails to lick off the fæces while still moist, or if the infective flea does not defæcate on the rat, no infection is brought about. A negative result of this kind is most likely to be attained when the number of infective fleas on the rat is very small, as seen in the large number of negative and small number of positive results in our series of experiments in which single fleas were used (see below, p. 661). That infective fleas in Cage J were rare is shown by the fact that between 23 : ix and 19 : x thirty fleas from this cage were dissected and examined without finding a single one infected. On the other hand, when fleas are sufficiently numerous and have been well infected, positive results are fairly certain (Experiment "C" of our preliminary report).

(v) Transmission is never effected until the Developmental Cycle is completed; that is to say, until at least Five Days have elapsed since the First Exposure of the Fleas to Infection.

We have found, as already stated, by direct observation, that the final form of the developmental cycle appears in the gut of the flea five days after the infective feed (see below, p. 630). We bring forward here a few instances to show that at least five days must elapse before the flea becomes infective, after having ingested trypanosomes from an infected rat.

(1) Experiment 20.—A cage colonised with forty-four fleas that had been exposed to infection from 4 : x : '09 to 8 : x : '09.

Rat 93 put into the cage from 8 : x to 12 : x, i. e. during a period in which the age of the infection in the fleas could not have been less

than two days old at the beginning nor more than eight days old at the end. Result negative.

(The next rat used in this experiment died, but subsequent rats used showed that the fleas had become infective.)

(2) Experiment 21.—A cage colonised with 157 fleas that had been exposed to infection from 11 : x : '09 to 15 : x : '09.

Rat 97 put in from 15 : x to 19 : x, i. e. during a period in which the age of the infection in the fleas could not have been less than a few hours at the beginning nor more than eight days at the end. Result negative.

(The next rat put in became infected, apparently about 27 : x; age of infection in the fleas then between twelve and sixteen days.)

(3) Experiment 22.—A cage colonised with 160 fleas exposed to infection from 24 : xi : '09 to 27 : xi : '09.

Rat 116 put in from 27 : xi to 30 : xi, i. e. during a period in which the age of the infection in the fleas could not have been less than a few hours at the beginning nor more than six days at the end. Result negative.

(The next rat put in became infected, apparently about 3 : xii; infection of the fleas then six to nine days old.)

(4) Experiment 23.—A cage colonised with 162 fleas exposed to infection from 7 : xii : '09 to 8 : xii : '09.

Rat 125 put in from 8 : xii to 11 : xii, i. e. during a period in which the age of the infection in the fleas could not have been less than a few hours at the beginning nor more than four days at the end. Result negative.

Rat 133 put in from 11 : xii to 13 : xii, i. e. during a period in which the age of the infection in the fleas could not have been less than four days at the beginning nor more than six days at the end. Result negative.

(The next rat put in became infected, apparently about 15 : xii; infection of the fleas then seven to eight days old.)

(5) Experiment 24.—A cage colonised with fifty fleas exposed to infection from 7 : xii : '09 to 8 : xii : '09.

Rat 126 put in from 8 : xii to 11 : xii, i. e. during a period in which the age of the infection in the fleas could not have been less than a few hours at the beginning nor more than four days at the end. Result negative.

(The next rat put in became infected, apparently about 3 : i : '10; age of the infection of the fleas then between twenty-six and twenty-seven days.)

(6) Experiment 25.—A cage colonised with seventy fleas exposed to infection from 31 : xii : '09 to 3 : i : '10.

Rat 152 put in from 3 : i to 6 : i, i. e. during a period in which the

age of the infection in the fleas was not less than a few hours at the beginning nor more than six days at the end. Result negative.

(The next rat put in became infected, apparently about 6 or 7 : i; the age of the infection at 7 : i was from four to seven days.)

(7) Experiment 45. Batch C (see below).—Bell-jar colonised with thirty fleas exposed to infection from 22 : vii : '13 to 23 : vii : '13.

No infection produced in rat 370 put in for a period of two days, during which the infection in the fleas could not have been less than five days old at the beginning nor more than seven days old at the end.

No infection produced in rat 370a, put in for a period of one day, during which the infection in the fleas could not have been less than eight days old at the beginning nor more than ten days old at the end.

(The next rat put in became infected.)

In the previous section it has also been pointed out that in Experiment 28, rat 209 escaped infection when exposed to infection by 138 fleas during a period of one day, at the beginning of which the age of infection in the fleas could not have been less than a few hours nor more than five days at the end. Rat 215 became infected by the fleas a day later, when the age of the infection in the fleas could not have been less than two or more than six days.

Putting together the results of this and the last section, it is seen that fleas in which there is a possibility, from the data of the experiment, of the infection being more than five days old, may fail to produce infection, although the subsequent history of those fleas shows them to have been infected effectively, but no infections have been obtained in any experiment of which the data are incompatible with the infection being at least six days old in the fleas that produced the infection.

(vi) The Infection of the Rat is brought about by the Small Trypanosome-form which is the Final Form of the Development.

This point is scarcely capable of direct proof, since it is impossible to be absolutely certain that when an infection has been produced no other forms of the developmental cycle in the flea were introduced into the rat except the trypanosome-forms. It can, however, be demonstrated in experiments planned for that purpose, that the trypanosome-forms

are present when an infection is produced. Nöller (1912) and Wenyon (1913), have shown that the trypanosome-forms were present in all cases in the fæces with which they infected rats per os.

The following experimental results indicate that the trypanosome-form is the effective agent in infection. In Experiment 35 B twelve fleas were taken at hazard from the infected breeding-cage and put on clean rat 243 for five days (23 : ii : '11 to 28 : ii : '11; Rat 243 was found to be infected on 3 : iii : '11). Ten of the fleas recovered were then dissected (the other two lost); of each flea the stomach was placed on one slide in a drop of salt-citrate solution, the rectum on another slide in another drop. Each stomach and each rectum were then teased up and examined microscopically in order to see if trypanosomes were present in any form; but since this examination had to be performed very rapidly and cursorily and without putting a coverslip over the drop, trypanosomes may have been often overlooked, when they were not present in abundance. Whether trypanosomes could be seen in the fresh specimen or not, each teased-up stomach was inoculated by means of a syringe into a separate clean rat; the rectum was only inoculated if trypanosomes were seen in it.[1] After the drop containing the teased-up stomach or rectum had been drawn up into the injecting syringe the film of moisture left on the slide was fixed with osmic vapour, stained with Giemsa's stain, and carefully searched for trypanosomes. The following are the results obtained with each flea.

Flea (1).—Nothing seen in the fresh stomach or rectum. Stomach inoculated into Rat 279. No infection produced. Nothing found in the preserved film.

Flea (2).—As last, stomach inoculated in rat 280, no infection, nothing found in the films.

[1] The reason for the differential treatment of the stomach and rectum was because we believed, at the time, that infection was brought about by regurgitation of infective trypanosomes through the proboscis from the stomach, and also because the presence of trypanosomes in the rectum is not so easily overlooked as in the stomach.

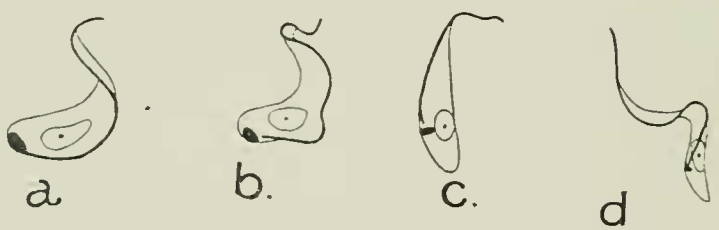
Flea (3).—As last, stomach inoculated into rat 281, no infection, nothing found in the films.

Flea (4).—One sluggish stumpy form, which may have been crithidial or trypaniform, was seen in the fresh teased-up stomach; nothing seen in the fresh rectum. Stomach inoculated into rat 283, result negative. Nothing found in the preserved film of the stomach.

Flea (5).—Nothing seen in the fresh stomach, numerous trypanosomes seen in the rectum. Stomach inoculated into rat 285, result positive. Rectum inoculated into rat 248, result negative. One trypanosome-form and one transitional form found in the preserved film of the stomach (Text-fig. 16, *b* and *c*). Nothing found in the preserved film of the rectum.

Flea (6).—Nothing seen in the fresh stomach; a few forms, some

TEXT-FIG. 16.



Small trypanosome-forms from the stomach-films of fleas 5 and 7 in Experiment 35 B, and flea 5 in Experiment 27 (see text). (× 2000.)

stout and of crithidial appearance and some slender, apparently trypaniform, seen in the rectum. Stomach inoculated into rat 286, result, negative; rectum not inoculated. No films preserved.

Flea (7).—Nothing seen in the fresh stomach or rectum. Stomach inoculated into rat 288, result positive. One trypanosome (Text-fig. 16, *a*) found in the preserved film of the stomach.

Fleas (8), (9), (10).—In each case nothing was seen in the fresh stomach or rectum. The stomachs were inoculated into rats 289, 262, 263 respectively, results in each case negative. Nothing found in the preserved films.

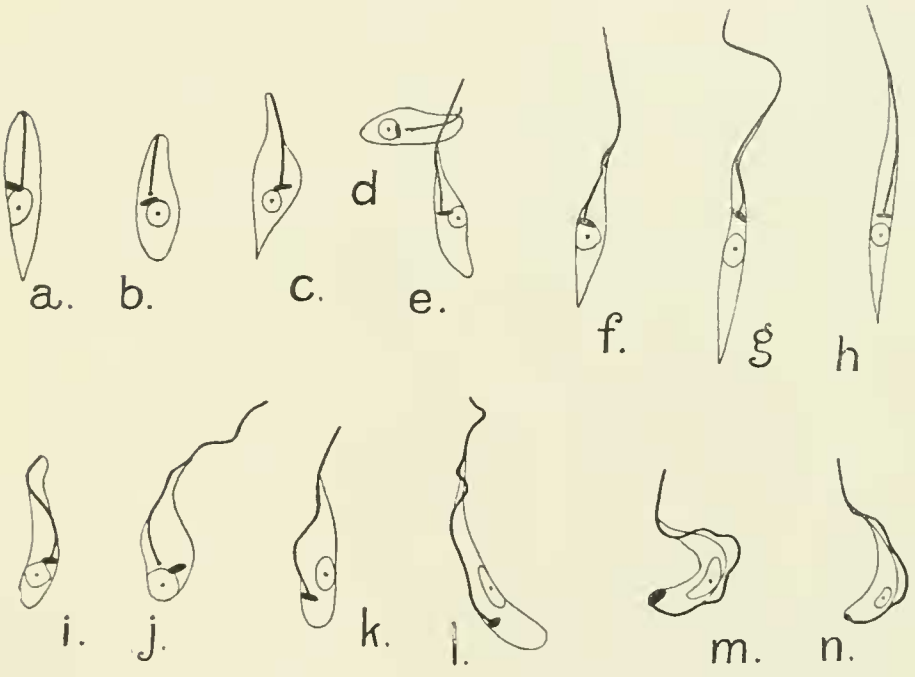
Summary.—In the case of two fleas out of the ten used, the stomachs, when inoculated into clean rats, produced an infection. The final trypanosome-stage was found in both the stomachs that produced infections, but in none of the remaining eight stomachs that produced no infection.

Experiments 27, 29 and 32 were conducted in a different

manner. Fleas taken from the infected breeding-cage were put each on a separate rat and left on it for three or four days. The flea was then recovered (if it could be found), dissected and examined.

Experiment 27.—Flea (5), placed on rat 207 for three days (2 : viii : '10 to 5 viii : '10) produced infection (see Table E). The flea dissected (5 : viii), and one large transitional form found in the slide of the stomach (Fig. 16, *d*).

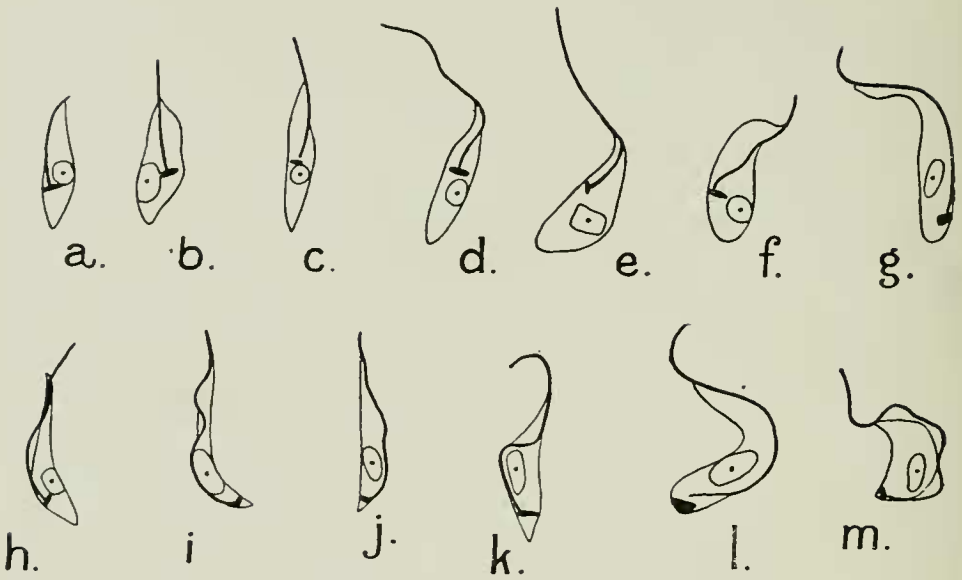
TEXT-FIG. 17.



Various forms (haptomonad, *a–d*, nectomonad, *e–h*, transitional, *i–l*, and trypaniform, *m* and *n*), from the stomach-film of flea 3, Experiment 29 (see text). (× 2000.)

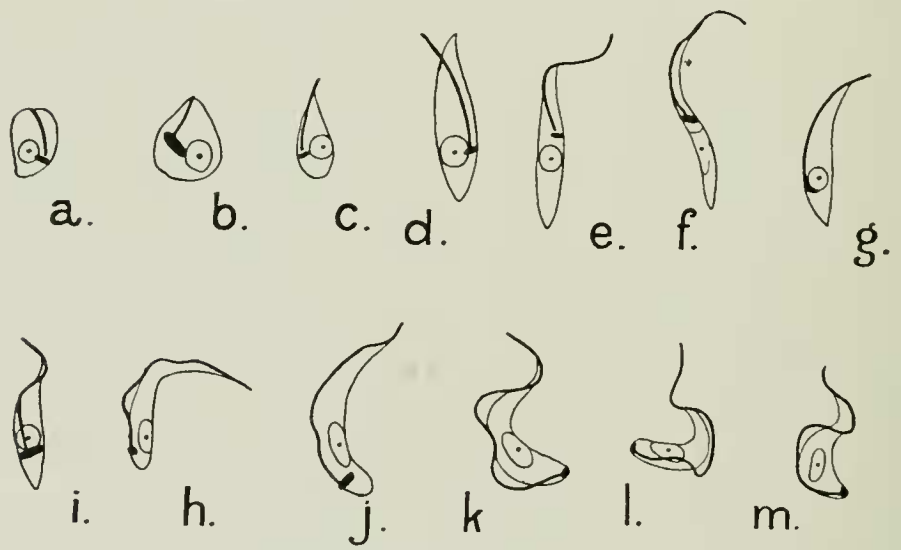
Experiment 29.—Flea (3) placed on rat 212 for three days, (19 : ix : '10 to 22 : ix : '10), recovered and dissected 22 : ix '10 (see Table E). Large clumps of attached forms were seen in the stomach and also free forms; nothing was seen in the intestine, rectum, salivary glands or proboscis. The preparations of the stomach showed crithidial, transitional and trypaniform types in abundance (Text-fig. 17). Rat 212 became infected and first showed trypanosomes in the blood on 28 : ix. Four other fleas in the same experiment failed to infect their rats; in two of these fleas nothing was found, in the third a small

TEXT-FIG. 18.



Various forms (haptomonad, *a*, *b*, nectomonad, *c*, *d*, transitional, *e–h*, and trypaniform, *i–m*), from the stomach-film of flea 3, Experiment 32 (g) (see text). (× 2000.)

TEXT-FIG 19.



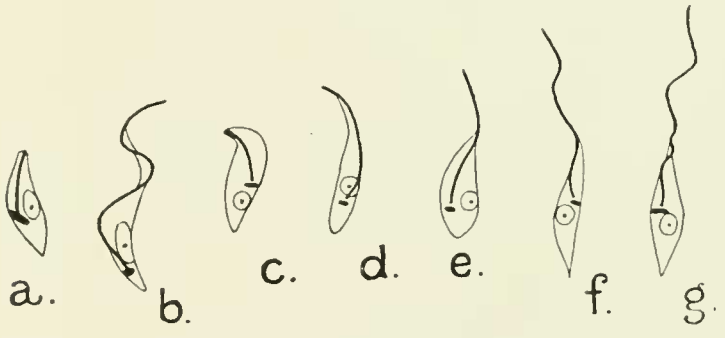
Various forms (haptomonads, *a–d*, nectomonad, *e*, transitional, *f–i*, and trypaniform, *j–m*) from the rectum-film of flea 3, Experiment 32 (h) (see text). (× 2000.)

attached clump was seen in the intestine, nothing in any other part of the flea. The remaining flea was not recovered.

Experiment 32 (g).—Flea (3) placed on rat 241 for four days (3 : xi : '10 to 7 : xi : '10) produced no infection. In the stomach of the flea, dissected 8 : xi, crithidial, transitional and trypaniform types were found (Fig. 18).

Experiment 32 (h).—Flea (3) placed on rat 244 for four days (11 : xi : '10 to 15 : xi '10) produced an infection (see Table E). The flea was dissected 15 : xi ; nothing was found in the stomach, intestine or salivary glands ; the rectum showed a typical swarming "pile-carpet" infection with all the usual types of form (Text-fig. 19).

TEXT-FIG. 20.



Flea 3, Experiment 32 (i). *a*, haptomonad, and *b*, trypanosome-form from the rectum-film; *c* and *d*, haptomonads, *e–g*, nectomonads, from the film of the intestine (see text). (× 2000.)

Experiment 32 (i).—Flea (3), placed on rat 247 for three days (12 : xi : '10 to 15 : xi : '10), produced no infection. The flea, dissected and examined (16 : xi), showed no trypanosomes in the stomach, but in the intestine were clumps attached behind the pylorus (Text-fig. 20, *c*), and the rectum contained a teeming infection (Text-fig. 20, *a* and *b*) of the usual types.

The cases cited show that when infections were produced the final trypanosome-form was found either in the stomach or rectum ; but it should also be mentioned that we have three instances in which the rat became infected under similar circumstances without our having been able to discover an infection of the flea, which must have been so scanty as to escape detection in our films. On the other hand the experiments also show that the infective form may be present in the

flea without any infection resulting when the flea is on the rat for not more than four days. The failure of the flea to infect in such cases must be correlated with the casual nature of the contaminative method of infection by the fleas, evidently not so sure a method as that of inoculation. It will be shown further (Experiment 39, below) that the period at which the flea becomes infective coincides with the first appearance of the small trypanosomes in the rectum.

(vii) The Final Infective Form of the Cycle is developed first in the Rectum on the Fifth Day of the Developmental Cycle, but may appear later in the Stomach.

In order to ascertain how soon the trypanosomes, ingested by the flea, attain to maturity in the different parts of the digestive tract of the flea an experiment (Experiment 39) was carried out in the following manner. A number of fleas (about one hundred) were collected from the non-infected breeding-cage, put into test-tubes, with clean sand, slightly damp, and kept there for four days (20 : iv : '11 to 24 : iv : '11), in order that they should be properly hungry and ready to feed. The fleas were then (24 : iv) put into a special flea-proof tin cage with a well-infected rat (No. 259).[1]

At regular intervals batches, each of ten fleas, were recovered from rat 259, kept in the test-tubes on sand, and dissected on the following day (to ensure that the fleas had not ingested blood containing trypanosomes for at least a day previous to being dissected). In the dissection of the fleas, the flea was first placed on a slide in a drop of salt-citrate solution and the proboscis removed by cutting through the head in the region of the eyes; the proboscis was then placed in a separate capsule in a small quantity of salt-citrate solution. Very often fæces were extruded when the

[1] Rat 259 was put in with a single flea on 22 : iii : '11; trypanosomes were first seen in the blood on 30 : iii; multiplication was ended on 4 : iv; see Table E, p. 617 above.

head was cut through. When this occurred the carcase of the flea was at once removed to another slide, and the extruded fæces examined microscopically. If trypanosomes were found in sufficient abundance the slide and coverslip were fixed and stained. The carcase of the flea was then opened in the hinder end of the abdomen, and the junction of stomach and intestine cut through behind the Malpighian tubes, after which the portion of the carcase containing the stomach was transferred to another slide. Then the stomach and Malpighian tubules, together with the proventriculus and œsophagus, were removed together and transferred to a second capsule, and the proctodæum (intestine with rectum) to a third. In making these dissections some of the contents of the stomach or rectum escaped on to the slide (the organs being purposely punctured to allow some contents to escape, when necessary). The escaped contents were examined microscopically, and if trypanosomes were found in them in sufficient numbers they were preserved.

After all the 10 fleas of each batch had been dissected in this way the 10 proboscides were inoculated into one clean rat, the 10 stomachs into another, and the 10 recta into a third. In each case the whole of the salt-solution in the capsule was injected also. The stomachs and recta were teased up as fine as possible, and the proboscides crushed up, before injecting them.

The following are the actual injections performed; in the results stated, 0 signifies that the rat inoculated acquired no infection, + that it became infected.

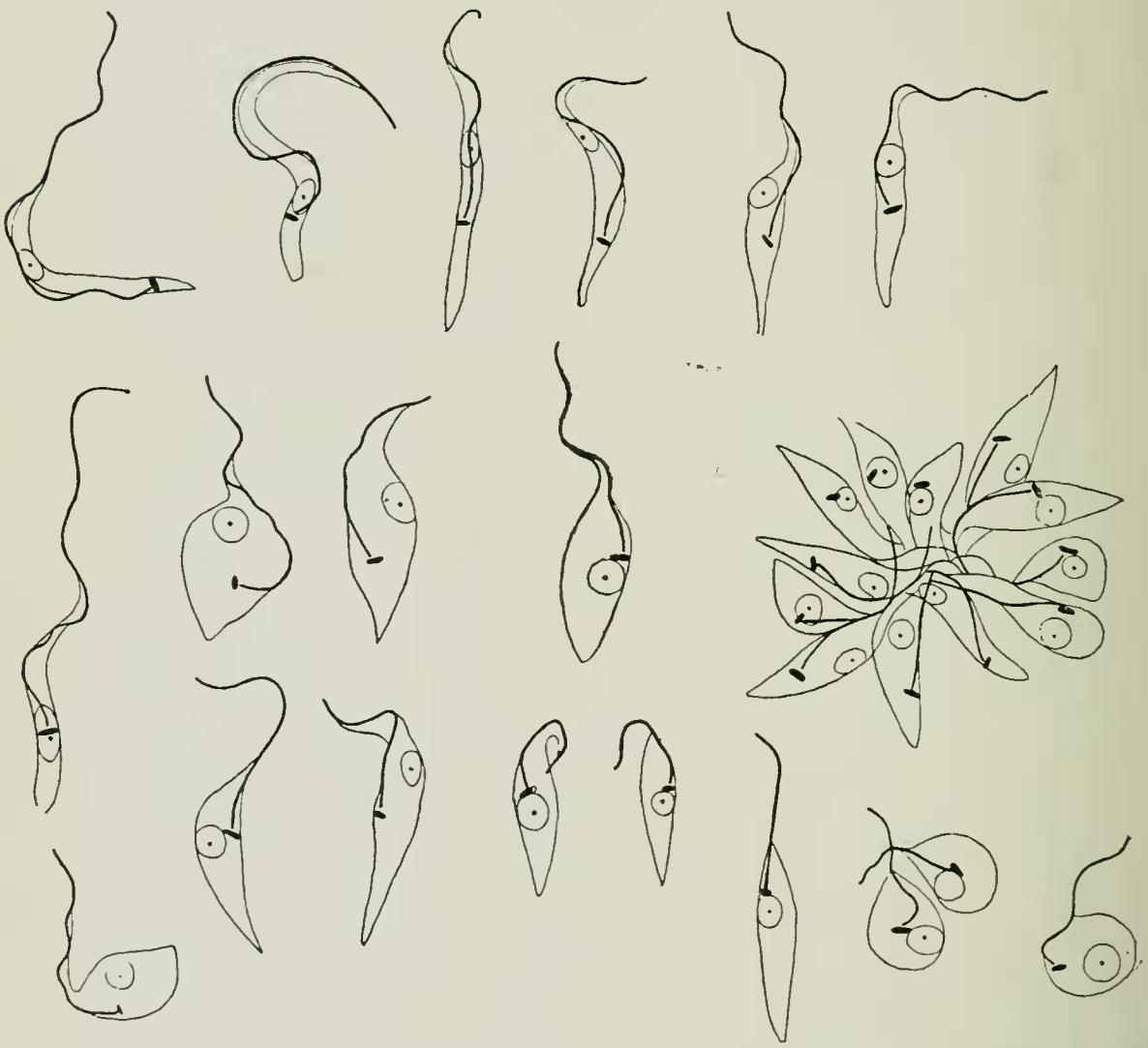
26 : iv : '11.—The 10 fleas recovered on the previous day were dissected; trypanosomes were seen in the stomach, rectum, and extruded fæces of several fleas. All trypanosomes seen appeared to be of quite ordinary type:

10 proboscides	injected	into	rat	289:	result 0
10 stomachs	,,	,,	,,	299:	,, 0
10 recta	,,	,,	,,	300:	,, 0

27 : iv : '11.—The 10 fleas recovered on the previous day were dissected; trypanosomes were seen in the stomach of one, the stomach and extruded fæces of another, in the stomach and rectum of a third, and in

the rectum and extruded fæces, very abundantly, of a fourth; the rectal forms appeared pear-shaped or club-shaped in the living state (Text-fig. 21), but no post-crithidial trypanosome-forms were present:

TEXT-FIG. 21.



Various forms from the rectum and fæces of a flea of the batch of 27 : iv : '11. Note that no final trypanosome-forms are present (see text). (× 2000.)

10 proboscides injected into rat 301: result 0
10 stomachs „ „ „ 302: „ 0
10 recta „ „ „ 303: „ 0

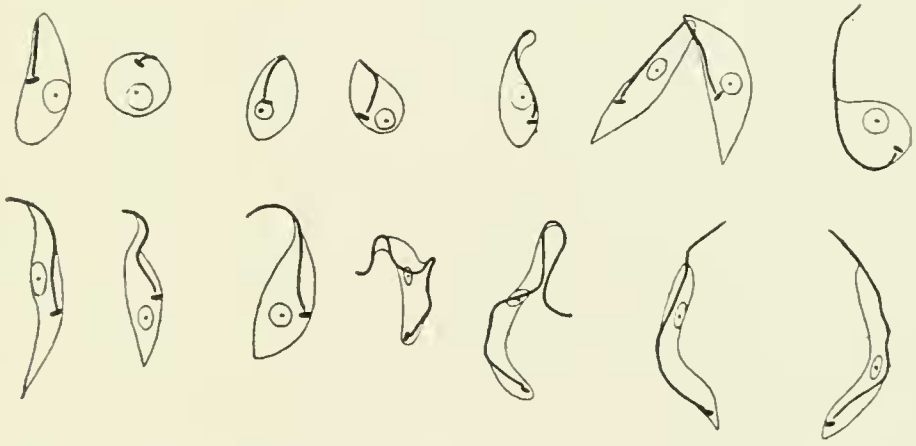
28 : iv : '11.—The 10 fleas recovered on the previous day were dissected; trypanosomes were seen in the stomachs of three, and in the stomachs and extruded fæces of two others. The fæces were preserved

in one case where they were seen, but no trypanosomes were found in the preparations:

10 proboscides injected into rat 304: result 0
10 stomachs „ „ „ 305: „ 0
10 recta „ „ „ 306: „ 0

29:iv:'11.—The 10 fleas recovered on the previous day were dissected; trypanosomes were seen in the extruded fæces of one, in the rectum and fæces of another (abundantly) and the rectum of a third (abundantly); of the last two, preparations were made of the rectal contents, and there were found pear-shaped crithidial, transitional, and post-crithidial trypaniform individuals (Text-fig. 22).

TEXT-FIG. 22.



Various forms from the rectum and fæces of two fleas of the batch of 29:iv:'11, Experiment 39 (see text). Note the trypanosome-forms (last 4 figs. to the right, second row). (× 2000.)

10 proboscides injected into rat 307: result 0
10 stomachs „ „ „ 308: „ 0
10 recta „ „ „ 309: „ +

1:v:'11.—The fleas recovered 29:iv were dissected; in one of them, a male, minute crithidial individuals were seen in the stomach contents, but not in the rectal contents; no preparation was made.

10 proboscides injected into rat 310: result 0
10 stomachs „ „ „ 311 „ 0
10 recta „ „ „ 312: „ +

4:v:'11.—The 10 fleas recovered on the previous day were dissected, trypanosomes were seen in the rectum of one, in the extruded fæces of another; no preparation made.

10 proboscides injected into rat 313: result 0
10 stomachs „ „ „ 314: „ +
10 recta „ „ „ 315: „ 0

Summary of Experiment 39.—None of the rats inoculated with organs of the fleas which had been exposed to infection two, three, or four days previously became infected. On the fifth and seventh days inoculation of the recta produced infections, while the inoculations of the stomachs were negative. On the tenth day, on the other hand, inoculation of the stomachs gave a positive, that of the recta a negative result. It is seen, therefore, (1) that the fleas first became infective on the fifth day, when also the post-crithidial trypanosomes were first found in preparations of the rectum; (2) that the developmental forms which produce infection were present in the rectum, but not in the stomach, on the fifth and seventh days; and in the stomach, but not in the rectum, on the tenth day.

(viii) The Developmental Forms of the Trypanosomes in the Flea are not infective when inoculated into the Rat during a period extending from a short time (half an hour?) after being taken up by the Flea until the Developmental Cycle is complete.

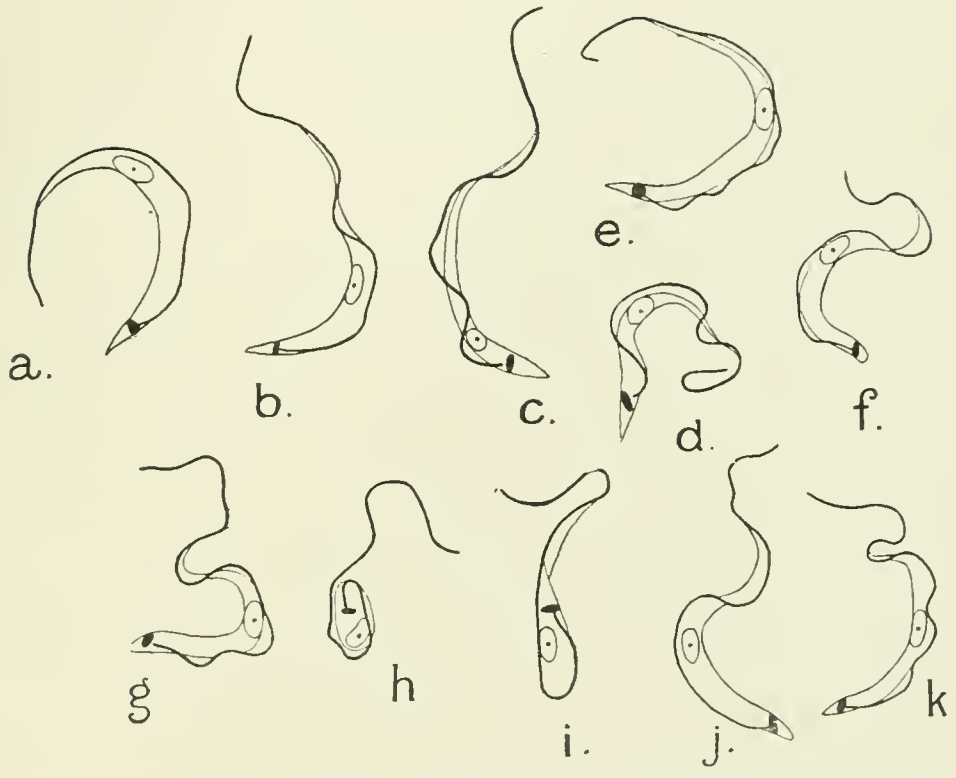
After we had shown, in Experiment 39 (see above), that the trypanosomes in the flea are not infective to the rat after they have been in the flea for two days, and that they do not acquire infectivity for five days after being ingested, we instituted a number of experiments with a view of discovering how soon the ingested trypanosomes lose their power of infecting.

In our first experiment a number of fleas collected from our non-infected breeding-cage and kept hungry for three days (7 : xi : '11 to 10 : xi : '11), were put on a well-infected rat at 6 a.m. (10 : xi : '11) and collected two hours later. They were then dissected in batches, the stomachs of each batch being placed together on the same slide, teased up in a drop of salt-solution, drawn up into an injection-syringe and inoculated into a clean rat. After the drop had been drawn up

into the syringe the film of moisture left on the slide was fixed with osmic vapour and stained with Giemsa's stain, in order to get an idea of the modification, if any, which the trypanosomes had undergone.

The following were the batches dissected and injected:

TEXT-FIG. 23.



Trypanosomes from the stomachs of fleas of the batches of 10 : xi : '11 (see text).

(1) Four fleas, injected into rat 323 at 11.35 a.m. The preserved film showed trypanosomes of the ordinary blood-type.

(2) Four fleas, injected into rat 324 at 12.35 p.m. The preserved film showed trypanosomes of the ordinary blood-type (Text-fig. 23, *a*, *b*).

(3) Four fleas, injected into rat 325 at 1.30 p.m. The preserved film showed trypanosomes for the most part unmodified, with *n* approximated to *N*, others dwarfed slightly as if beginning to degenerate (Text-fig. 23, *c*, *d*).

(4) Four fleas, injected at 2.35 p.m. into rat 326. The preserved film showed trypanosomes of the ordinary blood-type (Text-fig. 23, *e*, *f*).

(5) Four fleas, injected at 3.30 p.m. into rat 327. The preserved film

showed trypanosomes mostly modified, some rather lengthened out, others recurved (Text-fig. 23, *g*, *h*, *i*).

(6) Three fleas, injected at 4.35 p.m. into rat 327. The preserved film showed trypanosomes mostly unmodified, some rather long (Text-fig. 23, *j*. *k*).

All the results were negative, since none of the rats became infected. It is seen from the times of feeding and injecting the fleas that none of the trypanosomes had been in the fleas more than ten and half hours (6 a.m. to 4.35 p.m.), or less than three and half hours (8 a.m. to 11.30 a.m.).

After obtaining this result we made a number of other experiments, modifying slightly our method of procedure. Fleas taken from the non-infected breeding-cage were fed under observation on a well-infected rat and the time of feeding noted. The flea was recovered, dissected, and its stomach injected into a clean rat after being teased up in a drop of salt-citrate solution. As before, the film of moisture left on the slide was preserved in some cases.

In some cases also some blood from the infected rat was inoculated subcutaneously into a control clean rat. The controls were not always positive, however, since the subcutaneous method of injection is notoriously less efficient than the intra-peritoneal method, when a small quantity of blood is taken direct from the rat.

27 : xi : '11.—Fleas fed under observation on an infected rat after having been kept hungry for three days.

Fleas (1) and (2) fed at 11.35, injected into rat 329 at 12.10 (35 minutes). The preserved film showed trypanosomes quite unmodified.

Flea (3) fed at 11.45, injected into rat 329 at 12.20 (35 minutes). The preserved film showed trypanosomes quite unmodified.

Flea (4) fed at 11.30, injected into rat 330 at 12.30 (1 hour). Trypanosomes in preserved film quite unmodified.

Flea (5) fed at 11.50, injected into rat 330 at 12.50 (1 hour). Trypanosomes seen in the fresh stomach, but film not preserved.

Flea (6) fed at 11.50, injected into rat 330 at 12.50 (1 hour). Film badly preserved.

Flea (7) fed at 10.45, injected into rat 331 at 12.45 (2 hours). Trypanosomes in preserved film quite unmodified.

Summary.

Rat 329 was injected with 3 fleas 35 minutes after feeding.
„ 330 „ „ 3 „ 1 hour „ „
„ 331 „ „ 1 flea 2 hours after feeding.

The results in all three cases were negative. No control rat was injected.

8 : xii : '11.—Fleas fed under observation on an infected rat (322).

Fleas (1) and (2) fed at 11.40, injected into clean rat 324 at 12.26 (36 minutes). Active trypanosomes seen in the fresh stomachs.

Fleas (3) and (4) fed at 11·53, injected into rat 324 at 12.37 (44 minutes). Trypanosomes seen in the fresh stomachs.

Fleas (5), (6), and (7) fed at 12.4, injected into rat 324 at 12.42 (38 minutes). Trypanosomes seen in the fresh stomachs.

Fleas (8), (9), and (10) fed at 12.10, injected into rat 324 at 12.47 (37 minutes). Trypanosomes seen in the fresh stomachs.

Flea (11) fed at 12.20, injected into rat 324 at 12.55 (35 minutes).

Summary.—Rat 324 inoculated with the stomachs of eleven fleas fed on rat 322 between 35 and 44 minutes previously. Result negative.

Control rat 323 inoculated at the same time with a small drop of citrated blood from rat 322. Result positive (found to be infected on 15 : xii).

24 : i : '11.—Eight fleas, kept hungry for three days previously, were fed under observation on an infected rat (No. 392); the stomachs of six inoculated into clean rat 411.

Flea (1) fed 10 a.m, injected 11 a.m. (1 hour).
Flea (2) „ 10.10 a.m., injected 11.10 a.m. (1 hour).
Flea (3) „ 10.15 „ „ 11.14 „ (59 minutes).
Flea (4) „ 10.20 „ „ 11.20 „ (1 hour).
Flea (5) „ 10.25 „ „ 11.25 „ „
Flea (6) „ 10.29 „ „ 11.29 „ „

Fleas (7) and (8), fed 10.33 a.m. and 10.38 a.m. respectively, were dissected and fixed preparations made of them; numerous trypanosomes, quite unmodified in appearance, were found in them.

Summary.—Rat 411 inoculated with the stomachs of six fleas, each of which had fed on infected rat 392 an hour previously. Result negative.

Control rat 412 inoculated with a small drop of citrated blood from rat 392 at 12 noon. Result negative. (N.B.—Rat 412 put into the infected breeding-cage on 15 : ii : '13 contracted an infection in due course and was therefore not naturally immune.)

11: ii : '13.—Seven fleas fed on infected rat 402 under observation. The stomachs inoculated into clean rat 414.

Flea (1)	fed	10. 9	a.m.,	injected	11. 7 a.m.
Flea (2)	,,	10.12	,,	,,	11.12 ,,
Flea (3)	,,	10.17	,,	,,	11.17 ,,
Flea (4)	,,	10.25	,,	,,	11.25 ,,
Flea (5)	,,	10.28	,,	,,	11.28 ,,
Flea (6)	,,	10.37	,,	,,	11.38 ,,
Flea (7)	,,	10.58	,,	,,	11.58 ,,

(In all cases the time of feeding was reckoned from the moment the flea withdrew its proboscis.)

Some of the blood was allowed to escape from the stomach of flea (7) and permanent preparations made; numerous trypanosomes quite unmodified in appearance were found.

Summary.—Rat 414 acquired no infection; control rat 415, inoculated with one drop of blood from rat 402, became infected (trypanosomes first seen, 21 : ii : '13).

25 : ii : '13.—One flea was fed under observation at 10.45 a.m. on infected rat 415; the stomach was inoculated into clean rat 419 at 11.45. Results negative. (The other fleas refused to feed.)

27 : ii : '13.—Eight fleas fed under observation on infected rat 415; the stomachs inoculated into clean rat 419, in each case 59 minutes or an hour after feeding. Result negative.

Control rat 414 inoculated with a drop of blood from rat 415. Result negative (rat 414 was later infected by being put into the infected breeding-cage.)

It will be seen from the foregoing accounts that all experiments, in which infected blood ingested by a flea was inoculated into a clean rat from about half an hour onwards after ingestion by the flea, gave uniformly negative results. The controls were sometimes negative, sometimes positive.

(ix) The Flea, when once it has become infective, remains so for a considerable Length of Time.

This point was dealt with in our preliminary account (1910), when it was shown in two experiments ("C" and "D") that cages of fleas when once rendered infective continued to produce infections for some time without being re-infected. Experiment 22 ("C") was continued for some time after the publication of our paper, but on 28 : i : '10 an infected rat was unfortunately introduced into the cage, an oversight which vitiated all subsequent results, and the experiment was discontinued. During the period prior to this accident, however, the experiment was not open to objection, and produced a result which may be summarised as follows. A cage colonised with 160 clean fleas, into which an infected rat was introduced for three days (24 to 27 : xi : '09), and which produced the first infection between 30 : xi : '09 and 3 : xii : '09, continued to produce infections without being re-infected up to 24 : i : '10, a period of approximately 55 days. It is, therefore, a safe conclusion to affirm that the infectivity persisted in some fleas, or at least one flea, for that period of time.

Later a more exact experiment (Experiment 37) was carried out, in which single fleas were used. To begin with, 10 fleas were taken from the infected breeding-cage and put each on a clean rat for four days; at the end of that time the 10 fleas were recovered and each flea put by itself in a separate test-tube in damp sand for six days. Meanwhile the 10 rats were examined daily, and in due course two of them developed infection, the remaining eight being negative. The eight fleas that gave negative results were returned to the infected breeding-cage.

The two fleas that had been proved experimentally to be infective—henceforth known as Flea "A" and Flea "B"—were then placed singly on a succession of clean rats. That is to say, each flea was placed by itself on a clean rat for so many days, then was recovered and placed on another clean rat for so many days, and so on. The experiments with each

flea were continued until the flea disappeared: that is to say, until the flea could not be recovered when sought for.

The details and results of Experiment 37 have been summarised in tabular form in our preliminary communication (1911), but since we have frequently had occasion to refer to the experiment in the present memoir, we think it worth while to reproduce the table already published.

TABLE G.—Summary of Experiment 37.

Flea "A."

No. of rat.	Flea on the rat.	Result.	Trypanosomes.	
			First seen in blood.	Multiplication ended.
265	14–18 : ii : '11	+	24 : ii : '11	27 : ii : '11
249	24 : ii–1 : iii : '11	0		
260	1–6 : iii : '11	+	15 : iii : '11	(Rat died, 16 : iii : '11)
291	6–13 : iii : '11	0		
293	13–17 : iii : '11	0		
295	17–22 : iii : '11	+	27 : iii : '11	31 : iii : '11
249	23–27 : iii : '11	0		
291	27 : iii–1 : iv : '11	+	5 : iv : '11	9 : iv : '11
293	1–5 : iv : '11	0		
249	5–10 : iv : '11	0		
262	10–15 : iv : '11	0		
296	15–20 : iv : '11	+	22 : iv : '11	25 : iv : '11
263	20–25 : iv : '11	+	1 : v : '11	5 : v : '11
292	25–29 : iv : '11	+	1 : v : '11	6 : v : '11
293	29 : iv–4 : v : '11	0		
294	4–9 : v : '11	?	(Rat died, 9 : iv : '11)	
249	9–15 : v : '11	0		
	(15 : v : '11 flea not recovered.)			

Flea "B."

272	14–18 : ii : '11	+	24 : ii : '11	28 : ii : '11
259	24 : ii–1 : iii : '11	0		
253	1–6 : iii : '11	0		
292	6–13 : iii : '11	0		
294	13–17 : iii : '11	0		
296	17–22 : iii : '11	0		
259	23–27 : iii : '11	+	30 : iii : '11	4 : iv : '11
253	27 : iii–1 : iv : '11	+	5 : iv : '11	7 . iv : '11
292	1–5 : iv : '11	0		
294	5–10 : iv : '11	0		
	(10 : iv : '11 flea not recovered.)			

From the tabular summary it is seen that flea "A" remained infective from about 15 : ii : '11 to about 26 : iv : '11 —that is to say, for a period of 70 days—and flea "B" from about 16 : ii : '11 to about 27 : iii : '11—a period of 40 days. We have no data for determining the maximum period of time during which a flea can remain infective. It is, perhaps, not improbable that a flea, once rendered infective, may remain so as long as it lives, but we have no knowledge with regard to the average longevity of the flea. Flea "A" (♀) lived under our care from 14 : ii : '11 to 9 : v : '11—a period of 84 days; but we have no clue as to its age when it was first taken from the breeding-cage.

(x) The Trypanosome does not penetrate into the Salivary Glands of the Flea, but is confined, during its whole Development, to the Digestive Tract.

To prove this point we began by dissecting out salivary glands of fleas taken from the infected breeding-cage and examining the glands both in the fresh condition, by teasing them up or crushing them with a coverslip, in a drop of salt-citrate solution, and also by means of fixed permanent smears of the glands. In a large number of glands examined very carefully in this way many yeast-like organisms and other similar bodies were found, but never anything that appeared in the least like any possible stage of a trypanosome. Examination of fluid from the body-cavity gave negative results also.

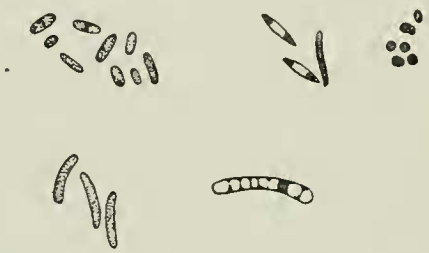
Thinking that we might have failed in these examinations to obtain an infective flea, we took fleas from the infected breeding-cage, put them singly on clean rats for three days or so, and then recovered and dissected them. The organs of the fleas were examined carefully in the fresh condition, and in some cases permanent preparations were also made of them and laid aside. If a rat became infected subsequently, and so proved the flea put on it to have been infective, the preparations of that flea were searched very carefully.

In this way we were able to examine the salivary glands and other organs of fleas which had been proved experimentally to be infective. The following are the details of the experiments and observations; the sign + signifies that trypanosomes were found in the organs mentioned, while 0 means that none were found:

(1) Flea (♀) put on rat 212 for three days (19 : ix : '10 to 22 : ix '10, Experiment 29). Rat 212 found to be infected 28 : ix; multiplication ended 30 : ix. Flea dissected 22 : ix; proboscis 0, salivary glands 0, stomach + (Fig. 17), rectum 0, intestines 0.

(2) Flea (♂) put on rat 223 for four days (10 : x : '10 to 14 : x : '10, Experiment 32a). Rat 223 found to be infected 17 : x; multiplication

Text-fig 24.



Yeast-like bodies of various kinds from the salivary glands of a flea. They are shown in groups, as they were found in the preparation. (× 2000.)

ended 22 : x. Flea dissected and examined 14 : x; no trypanosomes were seen in the proboscis, body-cavity, stomach, intestine, rectum or salivary glands; but unfortunately no permanent preparations were made.

(3) Flea (♀) put on rat 233 for five days (20 : x : '10 to 25 : x : '10, Experiment 32e). Rat 233 found to be infected 28 : x; multiplication ended 31 : x; scanty infection, with few trypanosomes. Flea dissected and examined 25 : x; no trypanosomes seen in proboscis, body-cavity, stomach, intestine, rectum or salivary glands; permanent preparations made of salivary glands, 0. (N.B.—Many yeast-like bodies in the salivary glands, see Text-fig. 24.)

(4) Flea (♂) put on rat 238 for three days (1 : xi : '10 to 4 : xi : '10. Experiment 32f). Rat 238 found to be infected 14 : xi; multiplication ended 16 : xi. Flea dissected and examined 4 : xi; no trypanosomes were seen in the proboscis, stomach, intestine, rectum or salivary glands; permanent preparations made of the stomach and salivary glands, both 0.

From the foregoing experiments it is seen that nothing which could be recognised as a trypanosome was found in the salivary glands of four fleas known to have been infective. In one of the four fleas the salivary glands were examined only in the fresh condition, but in the other three fleas the salivary glands were examined both in the fresh condition and in the permanent preparations.

It will be remarked, however, that no trypanosomes were found in any part of the digestive tract, when examined fresh, in three out of the four fleas, although there can scarcely be any doubt that trypanosomes must have been present. As a matter of fact, a scanty infection of the crithidial or final trypanosome-forms, small in size and sluggish in movement, is easily overlooked in the fresh preparations of the teased-up digestive tract, especially in an organ relatively so large as the stomach, which may be gorged with blood-débris greatly hindering and obscuring the examination. It has been our experience not infrequently that the smaller forms of the cycle have been found scantily in permanent preparations of stomachs in which nothing was seen in the fresh examination. This scarcely applies, however, to organs so minute as the salivary glands, the contents of which can be scanned comprehensively in one field of the microscope. We have, therefore, cited the second flea in spite of the fact that no permanent preparations of the salivary glands were examined.

After having made the negative observations recorded above, the idea occurred to us that any form of the trypanosome found in the salivary glands would probably be a final stage of the cycle, destined to be inoculated by the flea through the proboscis into the rat; and that consequently the examination of fleas which had produced an infection recently would be inconclusive, since in such fleas the salivary glands might be purged of their infection, temporarily at least.

We therefore carried out some experiments in which the object was to determine which organs of the flea contained the infective stages of the trypanosome, by dissecting fleas taken from the infected breeding-cage and injecting their

organs separately into clean rats. Thus a batch of fleas, at least five in number, was taken from the infected breeding-cage, and all the fleas in the batch were dissected at the same sitting. The stomachs of all the fleas were put together in one watch-glass and the salivary glands in another. Each flea has four salivary glands (two on each side of the body), but we did not succeed in every case in dissecting out all four of these minute organs; sometimes only two or three were obtained, or even one only of the four (on foggy mornings); but in every case at least one of the four glands was obtained. In the case of the recta (cut off behind the pylorus and therefore including the greater part of the intestine as well), each was examined microscopically and only kept for injection if seen to contain trypanosomes.

In our earlier experiment (Experiment 33) only the salivary glands were used for injection, with the following results:

TABLE H.—Experiment 33.

Date.	No. of fleas dissected.	No. of salivary glands injected.	Rat.	Result.
17 : xi : '13	11	34	248	0
5 : xii : '10	5	18	248	0

Hence fifty-two salivary glands, dissected out from sixteen fleas taken from the infected breeding-cage, failed to infect a clean rat when injected into it.

Our later experiment (Experiment 34) was carried out more elaborately. In the first place each batch of fleas, after having been collected from the infected breeding-cage, was first put on a clean rat, as a control. Then the fleas were recovered from the control rat and dissected; the salivary glands and stomachs were then injected into two clean rats respectively, and the recta, if anything was seen in them, into a third. The details of Experiment 34 are shown in the following table:

TABLE I.—Experiment 34.

No. of fleas.	Fleas put on control rat.	Fleas recovered from control rat.	Fleas dissected.	No. of salivary glands injected.	No. of rat (s. g.).	Result.	No. of stomachs injected.	No. of rat (stom.).	Result.	No. of recta injected.	No. of rat (recta).	Result.	No. of control rat.	Result control).
6	22 : xi : '10	25 : xi : '10	25 : xi : '10	24	256	0	6	257	0	1	255	0	239	0
6	12 : xii : '10	15 : xii : '10	15 : xii : '10	22	256	0	6	257	+	—	—	—	239	+
5	5 : i : '11	9 : i : '11	12 : i : '11	19	243*	0	5	245	+	—	—	—	231*	0
6	6 : i : '11	10 : i : '11	13 : i : '11	24	246	0	6	247	+	2	259	0	241	+
6	19 : i : '11	25 : i : '11	26 : i : '11	24	253*	0	6	259*	0	1	258	0	250	+
6	19 : i : '11	25 : i : '11	26 : i : '11	22	260	0	6	261	+	—	—	—	251	+
5	19 : i : '11	25 : i : '11	27 : i : '11	15	262	0	5	263*	0	—	—	—	252	+

The rats marked * became infected in subsequent experiments, and were therefore not naturally immune.

From the details summarised in the above table it is seen that no infection was produced by 150 salivary glands taken from forty fleas, divided into seven batches. In three of these batches, comprising eighteen fleas, from which sixty-eight salivary glands were obtained, both the control rats and the rats infected with the stomachs[1] became infected. In a fourth batch, comprising five fleas from which nineteen salivary glands were obtained, the control rat was negative, the rat injected with the stomachs was positive. In a fifth batch, comprising five fleas from which fifteen salivary glands were obtained, the control rat became infected but the stomachs produced no infection; the infectivity of this batch must have been in the recta, which unfortunately were not injected. Thus, omitting the two batches that gave negative results throughout and reckoning only with five batches proved to contain infective fleas, it is seen that 102 salivary glands obtained from twenty-eight fleas produced no infection. These results convinced us finally that the salivary glands of the flea play no part whatever in the transmission or development of the trypanosome, and from this time we paid no further attention to them.

(xi) The Rat can become infected by eating infected Fleas, but not until the Developmental Cycle of the Trypanosome in the Flea is completed.

The fact that rats can become infected by eating infected fleas must now be considered as well established. It was first stated in print by Strickland (1911), but was then already known to us both from experiments performed by ourselves and from others carried out by Dr. Nicoll at the Lister Institute (see our preliminary report, 1911).

We carried out some further experiments to determine whether the rat could become infected in this way before the

[1] It should be noted that the stomachs here included the post-pyloric upper end of the intestine, which, as shown above, is often the site where the trypanosomes establish themselves.

developmental cycle of the trypanosome was completed in the flea. The experiments (Experiment 46) were carried on for a long period, each experiment occupying a week. The following sample is typical of the whole series, all being performed in the same manner and each corresponding stage of the experiment being carried out on the same day of the week.

Sunday, 20 : iv : '13.—A large batch of fleas (batch 6) collected from the non-infected breeding-cage the Friday previously were put on a well-infected rat.

Tuesday, 22 : iv : '13.—About fifty fleas were collected from the batch (6) and fed to the "two-day rat," clean rat 445. The method was to place the fleas, when collected, on the surface of water in a suitable vessel : then each flea was carefully picked off with a fine forceps, and either it was decapitated on a slide in a drop of water with a needle, or its head was crushed with the forceps. Several fleas so treated were stuck into a pellet of damp bread and given to the rat, previously kept hungry for a time. As a rule the rats when fed in this way ate both the bread and the fleas readily and even greedily.

Thursday, 26 : iv : '13.—About fifty more fleas of the infected batch (6) were collected and fed to the "four-day rat," clean rat No. 448, in the same manner.

Saturday, 24 : iv . '13.—About fifty more fleas of the infected batch (6) were collected and fed to the "six-day rat," clean rat 432, in the same manner.

On the Sunday following a fresh batch (batch 7) was exposed to infection in the same way. On the Tuesday following the "two-day rat" (No. 445), being found not to have become infected, was fed again with about fifty fleas of batch 7. On the Thursday following the four-day rat (No. 448), not having become infected, was fed again with about fifty fleas of batch 7. On the Saturday following (3 : v : '13) the six-day rat (No. 432), which then showed no trypanosomes in its blood, was fed with thirty-four fleas of batch 7. Rat 432 was found, however, to be showing trypanosomes in its blood when examined four days later (7 : v : '13) ; it must have been infected by the fleas of batch 6. For batch 8, in the following week, a fresh clean rat, No. 452, was appointed to be the new six-day rat ; it later became infected by the six-day fleas of batch 12.

These experiments were continued in regular routine in the manner described, from 16 : ii : '13 to 22 : iii : '13, and from 20 : iv : '13 to 12 : vi : '13, in all thirteen weeks and

thirteen batches. The results may be summarized briefly: No rat fed either with fleas exposed to infection two days previously, or with fleas exposed to infection four days previously, became infected; on the other hand, two of the six-day rats fed with fleas exposed to infection six days previously became infected. It may be inferred, therefore, that rats cannot be infected by eating infected fleas until the infection in them is ripe, that is to say until the developmental cycle of the trypanosome in the flea is complete.

(It may be mentioned here that when any fleas remained over from any of the batches used in this experiment they were fed under observation on clean rats on the Monday, Tuesday or Wednesday following, that is to say, eight, nine or ten days after they had been first exposed to infection (see p. 654, below).)

(xii) Infection of the Rat is effected contaminatively, by way of the Rat's Mouth, by the Rat licking from off its Fur or Skin the Moist Fæces of Infective Fleas containing the Final Propagative Form of the Cycle.

This mechanism of infection was first demonstrated by Nöller (1912) and fully confirmed by Wenyon (1913) by means of experiments which put the matter beyond all reasonable doubt. Without repeating the experiments of these authors, we tested their results by another method, namely, by exposing rats, muzzled and pinioned, to infection by a large number of infected fleas.

For the purpose of our experiments, we made use in most cases of our infected breeding-cage, in which the fleas were swarming in great numbers, and in which an infected rat is kept habitually, so that the fleas ingest blood containing trypanosomes every time they feed. As a preparation for the experiment the infected rat was removed from the breeding-cage and kept apart, all fleas found on it being carefully cleaned off and returned to the breeding-cage. The breed-

ing-cage was then left without a rat in it for a certain time, sometimes merely from morning to evening, in other cases a day or two, in order to induce hunger in the fleas. Then a clean rat was introduced into the cage for a single night or from morning to evening, after having been muzzled in the following manner: The muzzle was a conical cap of fine wire gauze, with meshes too fine for a flea to pass through. The cap was large enough to cover the whole head, including the ears; the opening of the cone had a broad rim or sleeve of soft cloth, and a draw-tape was run through the free edge of the sleeve, so that by pulling the two ends of the tape the sleeve could be drawn up as tight as required. When fixing the muzzle, it was slipped over the head and then the sleeve was tightened round the neck behind the ears; the two free ends of the tape were then passed downwards and forwards over the chest and backwards under each axilla; each end of the tape was given a single turn round the upper joint of the fore-leg of its side and then passed backwards and upwards to be tied to the other end of the tape from the other side of the body over the back of the rat. In this way, not only was the head of the rat muzzled so that it could not lick itself or eat fleas, but owing to the fore-legs being secured firmly it could not use them to tear off its muzzle, which the rat always makes violent efforts to do, and which, in spite of all precautions, it sometimes succeeds in doing.

The clean rat, having been muzzled in the manner described, and exposed to the attentions of the fleas for a certain time, was removed from the breeding-cage, and before being unmuzzled it was subjected to a cleansing process which consisted of removing all fleas from it and of washing its fur all over thoroughly with a disinfectant (lysol, about 2 per cent.). The liquid used for washing usually became coloured reddish-brown from the fæces deposited on the fur by the fleas. After the rat had been cleansed thoroughly, its fur was dried by holding it close to an ordinary electric lamp and then its muzzle was removed and it was allowed to lick itself as much as it wished, being kept apart from all fleas and its blood examined at regular intervals.

As a variation of the above procedure the muzzled rat was not put into the infected breeding-cage in some instances, but into a bell-jar with a certain number of fleas taken from the infected breeding-cage and previously kept hungry.

At first the experiments were controlled by putting unmuzzled rats into the infected breeding-cage, but it was found superfluous to do this, since in many of our actual experiments the muzzled rats succeeded in tearing off their muzzles and thus furnished improvised but very efficient controls.

The following is a brief statement of the experiments (controls marked *) :

(1) Rat 447, muzzled and put into the infected breeding-cage from 4.30 p.m., 23 : iv : '13, to 10 a.m., 24 : iv : '13. Examined from 28 : iv to 30 : v, not infected ; inoculated later from wild rat and acquired infection in due course.

(2) Rat 449, muzzled, put into infected breeding-cage for night of 25–26 : iv : '13. Examined up to 30 : v, no infection ; put into infected breeding-cage, 30 : v, became infected in due course.

(3) * Rat 450, muzzled, put into infected breeding-cage evening of 30 : iv : '13 : found next morning with its muzzle off ; it became infected in due course.

After the above-mentioned experiments had been performed in the manner described, namely, by cleaning and disinfecting the rat before unmuzzling it, a change of procedure was adopted. The rat that had been muzzled and put in the infected breeding-cage was not disinfected when taken out, but, after having been freed from fleas, its fur was merely dried thoroughly by holding it near an electric lamp before unmuzzling the rat and allowing it to lick its fur. This was in order to see whether dried fæces would produce an infection when licked off.

(4) Rat 467, muzzled, put into infected breeding-cage, 8 a.m. to 4 p.m., 16 : ii : '14 (the infected rat having been removed from the cage two days previously). Examined up to 5 : iii, no infection.

(5) Rat 468, muzzled, put into infected breeding-cage 10 a.m. to 2 p.m., 19 : ii : '14. Procedure otherwise same as in last, no infection.

(6) *Rat 469, muzzled, put into infected breeding-cage, 7 p.m., 20 : ii : '14; found next morning (8 a.m.) with muzzle off; became infected in due course.

(7) Rat 470, muzzled, put into infected breeding-cage 11.30 p.m., 23 : ii : '14, treated as 467, etc.; no infection. Muzzled and put into bell-jar with 200 hungry fleas taken from infected breeding-cage 9 a.m. to 2.30 p.m., 3 : iii : '14; no infection.

(8) *Rat 472, muzzled, put into bell-jar with 200 hungry fleas from the infected breeding-cage, 9 a.m., 26 : ii : '14; found at 2.30 p.m. with its muzzle off; became infected in due course.

From the data quoted, it is seen that when the experiments were carried out successfully, that is to say, when the muzzle kept on, the rat did not become infected, alike whether its fur was cleaned and disinfected, or merely dried, before it was unmuzzled. But in those cases in which the rat succeeded in ridding itself of its muzzle by its own efforts, it became infected in due course.

It is evident that the sole effect of the muzzle is to exclude infection of the rat by way of its mouth. The way is still open for the rat to become infected through the skin, either (1) by the trypanosomes passing in through the puncture made by the proboscis of the flea, a possibility suggested by Nöller; or (2) by the fæces being rubbed into wounds or abrasions on the skin; or (3) by the small trypanosomes in the fæces penetrating by their own efforts through the skin. But since all the results with rats muzzled efficiently were negative, it is evident that no infection *per cutem* by any of these possible ways occurred in these experiments and it becomes highly probable that it does not take place naturally in any of these ways. On the other hand, since the muzzle excludes only infection *per os*, it becomes also probable that the rats that succeeded in tearing off their muzzles were infected in this manner.

It may be mentioned finally that an experiment (Experiment 43) was carried out in which the fæces, collected overnight in a moist glass capsule, of fleas from the infected breeding-cage were injected under the skin of a clean rat, but the result was negative.

(xiii) Can the Flea infect the Rat by inoculating the Trypanosomes into it through the Proboscis?

The first to throw doubt upon the occurrence of this mode of transmission were Strickland and Swellengrebel (1910, '12), who made a number of attempts to infect rats by feeding infected fleas on them through gauze. Every such experiment gave negative results. We repeated these experiments, and always with the same negative results.

We also carried out a large number of experiments in which fleas taken from the infected breeding-cage were fed on clean rats under observation. Our course of procedure, in its latest and most highly elaborate form, was as follows :[1]

A certain number of fleas, collected from the infected breeding-cage, or fed on an infected rat more than seven days previously, or known experimentally to be infective, were kept in a flask containing some damp sand for three or more days, to make them hungry. When the experiment was about to take place the fleas that it was proposed to use were put each into a separate test-tube. A clean rat was prepared for the experiment by shaving a small region of its skin, usually on the belly, sometimes on the inside of the thigh (a favourite spot for fleas to feed). The rat was then held still by an assistant, with its tonsure upwards. The test-tube was inverted on to the tonsure and at first kept pressed upon it; the flea was thus emptied out on to the shaved area of the skin. At first the flea runs round and round inside the circle formed by the rim of the test-tube, but usually comes to rest very soon, and inserts its proboscis into the skin. In some cases, however, the flea refuses to feed, and either continues to run about or remains perfectly still in one place, presenting a deceptive appearance

[1] We did not adopt Nöller's method of tethering the fleas, since it seemed to us better that the flea should be free and unhampered in its movements, and would then be more likely to feed in a natural way. Our colleagues, Dr. Martin and Mr. Bacot, who were doing experiments at the same time on transmission of plague, also found it unnecessary to tether the fleas.

of being engaged in feeding, but when recaptured and examined it is found to have been merely thinking.

In fleas carefully watched it is not difficult to observe with a hand-lens the penetration of the proboscis into the skin, the rush of blood into the stomach of the flea, and the withdrawal of the proboscis when the flea is replete. From a number of fleas that were timed carefully it was found that the male flea took about $1\frac{1}{4}$ minutes to fill its stomach, the female about $2\frac{1}{4}$ minutes. In all the fleas we have fed under observation we have never once observed the flea to defæcate while feeding, though particular attention was directed to this point. Only in one case, when the flea after feeding succeeded in making good its escape into the fur, it was found to have defæcated there.[1]

While the flea is feeding an assistant holds over it a paint-brush dipped in a thick syrupy solution of sugar and water. As soon as the flea has filled its stomach it withdraws its proboscis and makes a rush for the fur of the rat, but as soon as it does so the assistant dabs the paint-brush down on it and catches it. From the paint-brush, to which it sticks, the flea is put in water, which cleans off the sugar-syrup; it can then be dissected or put back in the cage, none the worse for its adventure. If it succeeds in getting into the fur of the rat it must be recaptured, and any fæces it may have deposited must be washed off with a disinfectant.

Fleas that refuse to feed can either be put back in their test-tubes and given another chance on the following day, or dissected and examined as controls.

In the majority of cases the fleas fed under observation were either fleas taken at random from the infected breeding-cage or fleas which had been fed on an infected rat at a

[1] Our experience of the feeding habits of Ceratophyllus fasciatus does not agree in the least with the account given by Nöller for Ctenocephalus canis. The habits of the two species are evidently quite different. The former lives in the burrows of the rat, and only goes on to the rat in order to feed, while the latter lives more or less permanently in the fur of the dog.

definite time, and of which the age of the infection was known exactly. In a few cases we used fleas which had been put singly on clean rats and had produced an infection, and were therefore known to be infective. Thus rat 367a had a "known infective" flea fed on it on 26 : vii : '12 and again on 29 : vii : '12, and another such flea was fed on the same rat on 3 : viii : '12, but the rat did not become infected (Experiment 44a).

We have in our notebooks records of 150 fleas fed on rats under observation in this way. Since the results were uniformly negative it is quite unnecessary to refer to them in further detail, but it may be of some interest to mention some cases in which the fleas were dissected and examined immediately after the experiment. These were fleas left over from the batches used in Experiment 46 (see above, p. 648), and were therefore, all of them, fleas in which the age of the infection was known.

11 : iii : '13.—Six fleas left over from batch 3; infection of the fleas nine days old. Five fleas fed, in three of which no trypanosomes were seen, in a fourth the stomach contained an infection of crithidial forms, and in a fifth both stomach and rectum contained crithidial forms. (In a sixth flea, which had not fed, both stomach and rectum also contained crithidial forms.)

12 : iii : '13.—Four fleas of the same batch as yesterday; infection ten days old. All fed. In two nothing was seen, the other two had crithidial forms in the rectum.

13 : iii : '13.—Two fleas of the same batch as last; infection eleven days old. One, which fed, had a few crithidial forms in the rectum. (In the other, not fed, nothing was found.)

17 : iii : '13.—Four fleas of batch 4; infection eight days old. One which fed, had a swarming infection of the rectum and a few crithidial forms in the stomach. (Of the three which did not feed all had crithidial forms in the rectum, one in the stomach also.)

18 : iii : '13.—Three fleas of the same batch as last; infection nine days old. One, which fed, had crithidial forms in the stomach. (Of the two which did not feed, in one nothing was found, the other had scanty crithidial forms both in stomach and rectum.)

19 : iii : '13.—Six fleas of the same batch as last; infection ten days old. Four fed. In one nothing was found; in two there were crithidial forms in the rectum only; in the fourth there were crithidial forms in

the stomach only. (Two did not feed. In one of them nothing was found, in the other there were crithidial forms in the rectum only.)

5 : v : '13.—Ten fleas of batch 8; infection eight days old. Five fed. Of these three showed crithidias in the rectum only, one in the rectum and stomach and one was quite negative. (Five did not feed. Of these one had crithidial forms in the stomach only, one in the rectum only, one both in the stomach and rectum, and two were quite negative.)

13 : v '13.—Six fleas of batch 8, infection nine days old. One fed, five did not; the examination of all the six was negative in result.

14 : v : '13.—Eight fleas of the same batch as last; infection ten days old. Five fed, three did not; the examination in all cases was negative in result.

19 : v : '13.—Six fleas of batch 9; infection eight days old. Only one flea fed: all the six negative.

20 : v : '13.—Six fleas of the same as last; infection nine days old. Four fed, two did not; all six negative.

26 : v : '13.—Six fleas of batch 10; infection seven and a half days old. One fed which was negative. (Of the five which did not feed, one was quite negative, three had crithidial forms in the rectum only, and one both in the stomach and rectum.)

27 : v : '13.—Six fleas of the same batch as last; infection eight and a half days old. Of four that fed, two were quite negative, two had crithidial forms in the rectum. (The two that did not feed had crithidial forms, both in stomach and rectum.)

2 : vi : '13.—Six fleas of batch 11; infection eight days old. Three that fed in two cases crithidial forms in the rectum only, the third was quite negative. (In the three that did not feed, two were quite negative, one had crithidial forms in the rectum only.)

3 : vi : '13.—Six fleas of same batch as last; infection nine days old. Two that fed were both negative. (In the four that did not feed, two had crithidial forms in the rectum only, one in the stomach only, one in both rectum and stomach.)

9 : vi : '13.—Seven fleas of batch 12; infection eight days old. Three fed, two of which had crithidial forms in the rectum only; one was quite negative. (All the four that did not feed had crithidial forms in the rectum, and one of them in the stomach also.)

It is seen from the data that in many cases the fleas that fed on the rats contained copious infections in the rectum, the stomach or both; but not in a single case was any infection produced in the rats fed upon by the fleas. At the present time, therefore, the answer to the question posed at the head of this section must be a very decided negative.

(xiv) Hereditary Transmission of the Trypanosome from Flea to Flea does not, in our Experience, take place.

In order to obtain, if possible, fleas infected hereditarily, a clean, freshly-prepared breeding-cage was colonised with 648 flea-larvæ taken from the infected breeding-cage at various dates between 23 : ix : '10 and 26 : x : '10. Adult fleas were first seen in the cage on the latter date, subsequently to which 100 more larvæ were added (3 : xi : '10).

Thinking, however, that the larvæ in the infected breeding-cage might possibly infect themselves directly from the fæces of adult fleas in the cage, we also colonised another clean breeding-cage with larvæ newly-hatched from eggs laid by fleas taken from the infected breeding-cage. The method was to take a certain number of fleas from the infected breeding-cage and keep them overnight in a glass capsule containing a glass coverslip at the bottom. In the morning a certain number of eggs were usually found, some on the glass of the capsule, some on the coverslip. The fleas having been returned to the infected breeding-cage, the eggs were carefully removed by means of a soft camel's-hair paint-brush, slightly moistened, from the glass of the capsule and each egg was placed on a small piece of black paper, to which it adhered. At first the eggs, attached either to the coverslip or to the black paper, were put in the new breeding-cage and allowed to hatch there; between 1 : x : '10 and 11 : x : '10 there were sixty eggs introduced in this way, but since they did not all hatch, another method was adopted. The eggs laid were kept in a glass capsule till they hatched, and then the newly-hatched larvæ were put into the breeding-cage. In this way 159 larvæ were introduced into the breeding-cage between 18 : x : '10 and 22 : xi : '10. The time taken by the eggs to hatch varied between six to eight days in October and ten to twelve days towards the end of November (laboratory-temperature).

In the two cages colonised in this way clean rats were kept

for a long time, but no infection was produced in either case.

(xv) The Trypanosomes in the Blood of the Rat can render Fleas infective very soon after they make their First Appearance in the Blood, before their Multiplication-Period is over.

In their paper on the life-history of Trypanosoma lewisi in the rat-louse, Breinl and Hindle (1909) state that they were unable to produce an infection of the louse when it was fed on the rat during the multiplication-period; they state that "during the first stages of infection, so long as dividing and segmenting forms were present in the blood, the trypanosomes taken up by the louse only degenerated." Wishing to find if this was true for the flea also, we did two experiments (Experiments 26 and 28) in which a number of fleas were first fed on an infected rat during the multiplication-period of the trypanosomes. The fleas were then recovered and used to colonise a freshly-prepared flea-cage, into which a clean rat was put. In both cases the result was positive, showing that fleas can become infective after having fed on infected rats in which the trypanosomes are undergoing multiplication.

We then planned and carried out a more elaborate experiment (Experiment 40) to determine how soon a rat infected by fleas can infect fleas again. For this purpose rat 317 was placed for one day (15–16 : v : '11) in the infected breeding-cage after the cage had been kept without a rat in it for three days, to make the fleas hungry. Rat 317 first showed trypanosomes in its blood on 21 : v; the multiplication-period was ended 25 : v or the following day.

Meanwhile, a succession of eight flea-cages, numbered A to H, were colonised with clean fleas, taken from the non-infected breeding-cage, and rat 317 was put in each cage successively for one day, thus :

Cage	A.	colonised	with	70	fleas,	12 : v ;	rat 317 put in	16–17 : v
,,	B	,,	,,	70	,,	13 : v	,, ,,	17–18 : v
,,	C	,,	,,	50	,,	15 : v	,, ,,	18–19 : v
,,	D	,,	,,	50	,,	16 : v	,, ,,	19–20 : v
,,	E	,,	,,	50	,,	17 : v	,, ,,	20–22 : v
,,	F	,,	,,	50	,,	18 : v	,, ,,	22–23 : v
,,	G	,,	,,	50	,,	19 : v	,, ,,	23–24 : v
,,	H	,,	,,	50	,,	20 : v	,, ,,	24–25 : v

After rat 317 had been taken out of each of the cages, a clean rat was put into the cage in its place and left in, with the following result :

Rat 218	put in	Cage	A,	17 : v ;	result	0	
,, 264	,,	,,	B	18 : v	,,	0	
,, 266	,,	,,	C	19 : v	,,	0	
,, 267	,,	,,	D	20 : v	,,	0	
,, 268	,,	,,	E	22 : v	,,	0	
,, 269	,,	,,	F	23 : v	,,	0	
,, 270	,,	,,	G	24 : v	,,	+	Found to be infected. 14 : vi.
,, 271	,,	,,	H	25 : v	,,	0	

From the above data it is seen that rat 317, exposed to infection 15–16 : v, did not render any fleas infective before the batch that fed on it in cage G, 23–24 : v, seven to nine days after it had been infected and two days after trypanosomes were first seen in its blood, at a time when the multiplication of the trypanosomes was proceeding actively. The late appearance of the infection in rat 270 (the record of which indicates that infection took place about 6 : vi, twelve days after it had been in contact with the fleas and thirteen days after the fleas themselves had been exposed to infection), indicates that only a small percentage of the fleas in cage G became infective.

From these experiments we deduce that infection of the flea is not dependent on the presence of special propagative forms of the trypanosome produced late in the rat's blood.

(3) Problems of Special Nature.

(xvi) The Trypanosomes succeed in establishing themselves in the Flea and rendering it infective to the Rat in only a Small Proportion of the Fleas (Ceratophyllus fasciatus) that ingest them.

It has already been pointed out above, in the description of the developmental cycle of the trypanosome, that in the greater number of the fleas fed on infected rats the trypanosomes degenerate and die out completely, and that they succeed in establishing themselves in but a small percentage of the fleas. We have also tested this question experimentally by the method of taking fleas from the infective breeding-cage and putting these fleas on clean rats. At first we carried out such experiments by taking small batches, each of five or six fleas, from the infected breeding-cage and putting each batch separately on a clean rat. Eleven such experiments were performed with the results summarised in Table J, from which it is seen that three batches, each of five fleas, produced one infection, and that eight batches, each of six fleas, produced four infections. In each case, as in many of the subsequent experiments now to be recorded, the fleas were left on the rat three or four days on the supposition, which is probably correct, that a flea will become sufficiently hungry to feed in the course of three days, and on the further supposition, which has now proved to be incorrect, that the infection passes into the rat through the proboscis of the flea.

The results of the experiments summarized in Table J are inconclusive, and could only permit of deductions approximately exact if carried out in great number. When a batch gives a positive result there is no clue as to the number of infective fleas contained in it. Further, it has been brought home to us by subsequent experience that an infective flea often fails to produce an infection. Thus, comparing Table J with Table I, it is seen that the batch of 5 : i : '11 failed to infect rat 231 (a rat infected subsequently in another ex-

TABLE J.—Experiments to determine the Percentage of Infective Fleas by taking batches of Fleas from the Infected Breeding-cage and putting each batch on a Clean Rat.

Experiment No.	Date.	No. of fleas in batch.	Fleas left on rat.	Rat No.	Result.	Tryps. first seen.	Multipl. ended.
27 bis	14 : ii : '10	5	4 days	186	0	—	—
,,	,,	5	4 ,,	187	+	21 : ii : '10	26 : ii : '10
,,	,,	5	4 ,,	188	0	—	—
34	22 : xi ; '10	6	3 ,,	239	0	—	—
,,	12 : xii : '10	6	3 ,,	239	+	19 : xii : '10	22 : xii : '10
,,	5 : i : '11	6	4 ,,	231	0	—	—
,,	6 : i : '11	6	4 ,,	241	+	16 : i : '11	19 : i : '11
,,	9 : i : '11	6	4 ,,	242	0	—	—
,,	19 : i : '11	6	4 ,,	250	0	—	—
,,	,,	6	4 ,,	251	+	26 : i : '11	30 : i : '11
,,	,,	6	4 ,,	252	+	25 : i : '11	30 : i : '11

Compare also Table I above (p. 645).

periment), but, nevertheless, the stomachs of this batch, injected into rat 245, produced an infection.

In order to obtain results from which more exact conclusions could be drawn, we experimented in a different manner, acting under the advice of Dr. M. Greenwood, Statistician of the Lister Institute. We took batches of fleas from the infected breeding-cage and put them on clean rats, one flea on each rat. A large number of such experiments were carried out with the results summarized in Table K, from which it is seen that 115 fleas placed each on a clean rat produced but eleven infections, a percentage of 9·56 approximately.

It is evident, however, that the bare numerical results of the experiments summarized in Table K cannot be taken as final, for the following reasons :

(1) The number of days given in the table as the time during which the flea was left on the rat is reckoned from the date the flea was put on the rat to the date on which the flea was sought for. But in many cases the flea when sought

TABLE K.—Infections produced by Fleas taken at Random from the Infected Breeding-cage and put singly on Clean Rats.

Fleas left on the rat.	Date put on.	Negative results.	Positive results.	Total negative.	Total positive.	Percentage of positive.
One day	6 : xii : '10	1	—	—	—	—
	12 : xii : '10	3	—	—	—	—
	18 : xii : '10	2	—	—	—	—
Total .	—	—	—	6	—	0
Three days	2 : viii : '10	4	1	—	—	—
	19 : ix : '10	2	1	—	—	—
	10 : x : '10	3	—	—	—	—
	1 : xi : '10	2	1	—	—	—
	18 : xi : '10	3	—	—	—	—
	19 : xi : '10	3	—	—	—	—
Total .	—	—	—	17	3	15
Four days	19 : ix : '10	2	—	—	—	—
	10 : x : '10	2	1	—	—	—
	13 : x : '10	3	—	—	—	—
	14 : x : '10	3	—	—	—	—
	3 : xi : '10	3	—	—	—	—
	11 : xi : '10	2	1	—	—	—
	12 : xi : '10	3	—	—	—	—
	29 : xi : '10	1	—	—	—	—
	14 : ii : '11	8	2	—	—	—
	28 : ii : '11	9	—	—	—	—
Total .	—	—	—	36	4	10
Five days	19 : x : '10	3	—	—	—	—
	20 : x . '10	2	1	—	—	—
Total .	—	—	—	5	1	16·6
Six days	27 : i : '11	10	—	—	—	—
Total .	—	—	—	10	—	0
Ten days	29 : vii : '11	7	1	—	—	—
Total .	—	—	—	7	1	12·5
Sixteen days	9 : i : '12	9	1	—	—	—
	13 : i : '13	7	1	—	—	—
Total .	—	—	—	16	2	11·1
Eighteen days	3 : iv : '13	5	—	—	—	—
	21 : iv : '13	2	—	—	—	—
Total .	—	—	—	7	—	0
Grand total .	—	—	—	104	11	9·56

for was not found, and it must be supposed that the flea died. If it died a natural death before going on to the rat the result would of course be negative whether the flea was infective or not. If eaten by the rat, an infective flea would probably produce a positive result.

(2) The fact, now established, that the flea does not infect by the puncture of the proboscis, but contaminatively, renders the infection a very casual affair, especially when only one flea is on the rat, and the probability of the infection taking place is relatively low. This is clearly shown by Experiment 37, tabulated above (Table G, p. 640), in which one infective flea, put on seventeen rats successively, over a period of about three months, infected seven of them, and another flea, put on ten rats over a period of about two months, infected only three of them. An analysis of these results is given in Table L, from which it is seen that twenty-seven exposures of rats to infection by two fleas known to be infective produced ten infections, equivalent to 37 per cent. of positive results.

TABLE L.—Infections produced by Two Fleas known experimentally to be Infective and placed singly on Clean Rats.

Flea left on the rat.	Negative results.	Positive results.	Total negative.	Total positive.
Four days . . .	4	4		
Five days . . .	10	6		
Six days . . .	1	—		
Seven days . . .	2	—		
			17	10

A side-light on the problem of the percentage of infective fleas might be obtained from the dissection and examination of fleas six days or more after they have been fed on an infected rat. From Table B it is seen that, of 118 such fleas examined, fifty-three were found to contain stages of the trypanosome; of these twenty-eight had a scanty, twenty-five

an abundant, infection. The value of these data, however, is somewhat uncertain, as an aid to the solution of the problem under consideration.

From the foregoing summary of the data, it is evident that the computation of the percentage of fleas that become infective, of those exposed to infection, is a somewhat complicated statistical problem. We have submitted the data to Dr. Greenwood, who has kindly supplied us with the report appended below, from which it is seen that the percentage of infected fleas lies probably between 5·9 per cent. and 45·7 per cent., the mean being 25·8 per cent.

Report of Dr. M. Greenwood.

The problem it is desired to solve is the following:

Within what limits does the true proportion of infective fleas in the population of which those enumerated in Table K are a sample probably lie?

Of 115 fleas left not more than eighteen days on clean rats, eleven produced infections. But all infective fleas do not produce infections, and, according to Table L, in twenty-seven trials with unquestionably infective fleas only ten produced infections in clean rats. Accordingly, it follows that the proportion of fleas in the first experiment which actually produced infection must be divided by the proportion found in the other experiment to arrive at the ratio of potentially infective fleas, which is the quantity sought. This is—

$$\frac{\frac{11}{115}}{\frac{10}{27}} = \cdot 2583 \text{ or } 25\cdot 8 \text{ per cent.}$$

But the two ratios from which this result is derived are each subject to errors of random sampling, and it is necessary to compute the "probable error" of sampling to which the final proportion is subject. Since the two proportions are entirely independent one of another, the square of the standard deviation of their ratio is $\frac{s_A^2 . B^2 + s_B^2 . A^2}{B^4}$ where A

is the proportion observed among the 115 fleas, and s_A its standard deviation and B and s_B similar quantities in the case of the twenty-seven trials of the other experiments.

Using this formula we reach 9·84 per cent. as the value of the standard deviation or ·67449 times this = 6·64 per cent. for the "probable error." Taking the usual margin, three times the "probable error," the conclusion may be drawn that the real proportion of infective fleas is very unlikely to be beyond the limits 5·9 per cent. and 45·7 per cent.

This is the conclusion which might, I think, legitimately be drawn from the two experiments, but two cautions must be had in mind. The first is that the number of trials in the second Experiment, 27, is rather small, and consequently the application of the customary theory of sampling errors must be made with hesitation. The second caution is that we are using twenty-seven trials with two fleas, not twenty-seven separate fleas, consequently, the two experiments are not strictly in pari materia. The two fleas used gave very different proportions of successes, and it might happen that were a larger number of definitely infective fleas used, the factor for division would be substantially modified. The above calculation can naturally give us no information on this point since it proceeds in terms of trials, twenty-seven trials with two fleas being assumed to be the same as twenty-seven trials with twenty-seven fleas, and that the differences do not depend upon the idiosyncrasies of the fleas, but upon the fluctuations of chance, the fleas being used simply as dice or counters.

(xvii) Can the First Phase of the Development of the Trypanosomes, namely, the Intra-Cellular Multiplication in the Stomach of the Flea continue beyond the Second Feed of the Flea (counting as the First Feed that by which it became infected)?

With regard to this point, it should first be made quite clear that our observations on fleas examined during early

periods of the development show conclusively that the trypanosomes may have disappeared from the stomach, and the rectal-phase may be well started, even so early as 18, 24, or 36 hours after the first feed.[1] A very clear case of this is the stomach mentioned above (p. 555), in which the infection was thirty-six hours old, and which was examined after being cut into a series of sections; no trypanosomes of any kind were found in the stomach, but immediately behind the pylorus were two attached clumps of quite normal crithidial type. On the other hand, in fleas not fed again after the infective feed we have found normal forms of the stomach-phase as late as three, four, or even five days after the first feed. From such observations it is evident that the stomach-phase is of very variable duration, for some reason, and that in some cases it is ended[2] long before the time when the flea would, under natural conditions, feed again, while in other cases it persists at least up to this time.

The question, therefore, is not, "Does the stomach-phase continue beyond the second feed?" (since it is certain that it is very often ended before the second feed), but "Can it do so?" and with regard to the question so posed it must be pointed out that a single clear instance of the stomach-phase persisting beyond the second flea would suffice to give an answer in the affirmative with certainty; but so long as the

[1] We do not refer to those cases in which the trypanosomes had disappeared from the stomach by degeneration, and in which the rectum was either empty or contained only degenerative forms; but only to those cases in which the presence of true crithidial forms in the intestine or rectum showed that the development of the trypanosome was following its normal course.

[2] Assuming, that is, that the intracellular multiplication is an essential part of, and takes place invariably in, the normal development of T. lewisi; we believe this to be the case, but we are unable to assert that it is so. It is at least within the bounds of possibility that the development may take occasionally a short cut, that is to say, that the trypanosomes may pass on to the rectum, and there establish the normal crithidial phase without undergoing intracellular multiplication in the stomach.

TABLE M.—Experiments to Test the Influence of a Second Feed on the Persistence of the Stomach-phase of the Trypanosomes in the Flea.

A.—Fleas not Fed a Second Time.

	Date of first (infective) feed.	Date of dissection of flea.	Age of infection in the flea.	Contents of stomach.	Contents of rectum.
1.	24 : vi : '12	27 : vi : '12	3 days	A few active trypanosomes of developmental type	Nothing.
2.	..	..	..	Many active trypanosomes and intracellular stages	A few forms, apparently degenerative
3.	..	..	..	A few active trypanosomes and many intracellular stages.	Two seen, apparently degenerative forms.
4.	..	..	..	Trypanosomes free and intracellular	Nothing.
5.	..	..	..	Nothing	Small forms, apparently degenerative.
6.	..	..	..	A few sluggish trypanosomes, apparently degenerative	Small forms, apparently developmental.
7.	..	..	..	Trypanosomes free and intracellular	Developmental crithidial forms.
8.	..	..	..	Nothing	Nothing.
9.	..	..	..	A few free trypanosomes . .	A few small forms and one "tadpole."
10.	..	..	..	Nothing	A few small forms, apparently developmental
11.	..	28 : vi : '12	4 days	.. . . . .	Developmental crithidias.
12.	..	..	..	.. . . . .	A few small forms of crithidial appearance.
13.	..	..	..	.. . . . .	Nothing.
14.	..	..	..	Trypanosomes free and intracellular	A few crithidial (?) forms.
15.	..	..	..	Nothing	Nothing.
16.	..	..	..	.. . . . .	Crithidias fairly numerous.
17.	..	..	..	A few trypanosomes free and intracellular	Crithidias fairly numerous.

18.	1 : vii : '12	3 : vii : '12	2 days	Nothing	Degenerative forms.
19.	,,	,,	,,	,, ...	Nothing.
20.	,,	,,	,,	,, ...	Degenerative forms.
21.	,,	,,	,,	,, ...	Nothing.
22.	,,	,,	,,	,, ...	Developmental crithidias.
23.	,,	4 : vii : '12	3 days	,, ...	Degenerative (?) forms.
24.	,,	,,	,,	A few free and intracellular trypanosomes	Degenerative (?) forms.
25.	,,	,,	,,	Nothing	Nothing.
26.	,,	,,	,,	,, ...	Degenerative (?) forms.
27.	,,	,,	,,	A few free trypanosomes . .	Nothing.
28.	,,	,,	,,	A few small forms	Swarms of crithidias.
29.	,,	5 : vii : '12	4 days	Nothing	Nothing.
30.	,,	,,	,,	,, ...	,,
31.	,,	,,	,,	One long trypanosome seen . .	Crithidial (?) forms.
32.	,,	,,	,,	Active free trypanosomes . .	Two degenerative (?) forms.
33.	,,	,,	,,	Nothing	Nothing.
34.	,,	,,	,,	A few long trypanosomes . .	Two active slender forms.
35.	8 : vii : '12 (night)	11 : vii : '12	2½ days	A few trypanosomes . . .	Nothing.
36.	8 : vii : '12	,,	,,	Nothing	A few degenerative forms.
37.	,,	,,	,,	Long free trypanosomes and attached clumps	A few attached pointed forms.
38.	,,	,,	,,	Nothing	Crithidial forms.
39.	,,	,,	,,	A few trypanosomes . . .	Developmental (?) crithidias.
40.	,,	,,	,,	Nothing	Crithidial forms.
41.	,,	,,	,,	,, ...	Crithidial (?) forms.
42.	,,	,,	,,	,, ...	Clump of pointed forms.
43.	,,	12 : vii : '12	3½ days	,, ...	Crithidias swarming.
44.	,,	,,	,,	,, ...	Crithidias.
45.	,,	,,	,,	Trypanosomes free and intracellular	Nothing.
46.	,,	,,	,,	Nothing	,,
47.	,,	,,	,,	,, ...	One developmental form.
48.	,,	,,	,,	,, ...	Nothing.
49.	,,	,,	,,	Free trypanosomes	,,

TABLE M—(*continued*).

B.—Fleas Fed a Second Time.

	Date of first (infective) feed.	Date of second feed.	Date of dissection of flea.	Age of infection in the flea.	Contents of stomach.	Contents of rectum.
1.	1 : vii : '12	3 : vii : '12	4 : vii : '12	3 days	Nothing	Nothing.
2.	,,	,,	,,	,,	,, ...	,,
3.	,,	,,	,,	,,	,, ...	,,
4.	,,	,,	,,	,,	,, ...	,,
5.	,,	,,	,,	,,	,, ...	Crithidial forms.
6.	,,	,,	,,	,,	,, ...	Nothing.
7.	,,	,,	5 : vii : '12	4 days	,, ...	,,
8.	,,	,,	,,	,,	,, ...	,,
9.	,,	,,	,,	,,	,, ...	,,
10.	,,	,,	,,	,,	,, ...	,,
11.	,,	,,	,,	,,	,, ...	,,
12.	,,	,,	,,	,,	,, ...	,,
13.	8 : vii : '12 (night)	11 : vii : '12 (9–10 a.m.)	11 : vii : '12	$2\frac{1}{2}$ days	,, ...	Degenerative (?) forms.
14.	8 : vii : '12	11 : vii : '12	,,	,,	One or two small pear-shaped forms (post-pyloric (?))	A few crithidial forms.
15.	,,	,,	,,	,,	Nothing	Nothing.
16.	,,	,,	,,	,,	,, ...	Crithidial forms.
17.	,,	,,	,,	,,	,, ...	Nothing.
18.	,,	,,	,,	,,	,, ...	Degenerative (?) forms.
19.	,,	,,	12 : vii : '12	$3\frac{1}{2}$ days	,, ...	Crithidial forms.
20.	,,		,,	,,	,, ...	Nothing.
21.	,,	,,	,,	,,	,, ...	A few crithidias.
22.	,,	,,	,,	,,	,, ...	A few degenerative (?) forms.
23.	,,	,,	,,	,,	,, ...	Crithidias.
24.	,,	,,	,,	,,	,, ...	A few pointed forms.
25.	,,	,,	,,	,,	,, ...	Pointed forms.

26.	8 : vii : '12	11 : vii : '12	12 : vii : '12	3½ days	Nothing	A few pointed forms.
27.	,,	,,	,,	,,	,, ...	Nothing.
28.	24 : vi : '12	27 : vi : '12	28 : vi : '12	4 days	,, ...	,,
29.	,,	,,	,,	,,	,, ...	,,
30.	,,	,,	,,	,,	,, ...	Numerous crithidias.
31.	,,	,,	,,	,,	,, ...	Nothing.
32.	,,	,,	,,	,,	,, ...	A few crithidial (?) forms.

N. B.—When a query (?) is affixed to the statement concerning the contents of the rectum, it signifies that the determination of the nature of the forms seen in the fresh state was not quite certain and that it was not confirmed by the examination of permanent preparations.

ANALYSIS OF THE RESULTS.

Series A.

No trypanosomes seen in 11 fleas.

Trypanosomes seen only in the rectum in 18 fleas; in 4 of the cases doubtfully, in 3 certainly, of degenerative type; in 3 of the cases doubtfully, in 8 certainly, of developmental type.

Trypanosomes seen only in the stomach in 6 fleas.

Trypanosomes seen both in the stomach and rectum in 14 fleas; the rectum contained in 5 of these fleas forms doubtfully of degenerative type; it contained in 4 of them forms doubtfully, in 5 of them forms certainly, of developmental type.

Series B.

No trypanosomes seen in 18 fleas.

Trypanosomes seen only in the rectum in 13 fleas; in 6 of these fleas doubtfully of degenerative type; in 1 doubtfully, in 6 certainly, of developmental type.

Trypanosomes seen both in stomach and rectum in 1 flea; in this case all the forms seen were crithidial in character, and those seen in the teased-up stomach may have been post-pyloric in position.

Summary.

A.—In 49 fleas not fed a second time, trypanosomes were found in the stomach in 20 cases, and were not found in 29 cases, in 11 of which they appeared to have disappeared altogether in the flea.

B.—Counting only those cases in which the rectum certainly contained developmental forms—namely, 7 fleas—the typical stomach-forms were absent in all.

answer is in the negative, it cannot be regarded as certain, but only as possessing a greater or less degree of probability.

In order to test this point we fed batches of fleas on infected rats and then divided each such batch usually into two batches, A and B. Batch A in each such case was kept starved until it was examined; batch B was fed again before being examined. In cases where fleas of batch B were found on examination not to have availed themselves of the chance of feeding, they were reckoned in batch A. Sometimes the original batch was not divided, but treated as a whole either as an "A" (not re-fed) or "B" (re-fed) batch.

The results of these experiments are tabulated in Table M, which seems at first sight decidedly in favour of the conclusion that the trypanosomes cannot persist beyond the second feed of the flea. It is seen that in forty-nine fleas not fed again after the infective feed, trypanosomes were present in twenty cases; in thirteen of the cases the trypanosomes were of the long stomach type and in eight cases intracellular forms were seen. On the other hand, in thirty-two fleas examined after having been fed a second time, the typical multiplicative stomach-phase was not present in a single instance. Unfortunately, the force of these figures is rather weakened by the fact that, of the fleas fed a second time it can only be asserted positively in seven cases that the rectum contained true developmental crithidial forms and that the developmental cycle was in these cases a "going concern," so to speak. While the figures make it probable, to a certain degree, that the stomach-phase, if it persists up to the time of the second feed, must come to an end then, this conclusion cannot be considered established and must remain a point for further investigation.

If it be true that the stomach-phase cannot persist beyond the second feed, we may enquire how such a result is brought about. It is intelligible that a fresh meal of blood might sweep on all free, extracellular trypanosomes from the stomach towards the rectum, but this would not account for the disappearance of the intracellular forms. It has been

mentioned above that in some insects the epithelium of the mid-gut is regenerated completely after each meal, and we stated further that we were not in a position either to affirm or to deny that, in the case of the flea, the regeneration of the stomach-epithelium takes place in regular correlation with the feeding. If it were so, however, it would become quite intelligible why a second feed should put an end to the intracellular multiplication in the stomach.

(xviii) Starvation of the Flea during the Incubation Period of the Cycle does not inhibit, nor does it necessarily retard, the Developmental Cycle of the Trypanosome in the Flea.

Experiment 45.—A batch of about 250 clean fleas, having been collected from the non-infected breeding-cage and kept hungry for three days, were put (22 : vii : '13) on a well-infected rat, for about twenty-four hours. The next day 150 of the fleas were recovered and kept in a flask containing some damp sand.

Two days later (25 : vii), thirty of these 150 fleas in the flask were put into a freshly-prepared bell-jar A with a clean rat 368. The next day (26 : vii) rat 368 was removed from bell-jar A, all the fleas on it being cleaned off carefully and put back into the bell-jar. Rat 368, examined regularly up to 26 : viii, did not become infected.

Two days later (28 : vii), clean rat 368a was put into bell-jar A, containing the fleas that had been in contact with rat 368. On the same day, thirty more fleas from the flask were put into bell-jar B with clean rat 369; another thirty in bell-jar C with clean rat 370; and another thirty in bell-jar D with clean rat 371. It will be remembered that the fleas in the flask had been exposed to infection for one day (22–23 : vii), and kept without food since then; consequently, bell-jars B, C, and D were colonised each with thirty fleas that had been exposed to infection between five and six days previously and starved since then.

The next day (29 : vii), rat 368a was removed from bell-jar A and all fleas recovered from it put back into the bell-jar. Rat 368a did not acquire infection. The same day rats 369, 370, and 371 were removed from bell-jars B, C, and D, all fleas being recovered from them and put back into the respective bell-jars. Rats 369 and 370 did not become infected; rat 371, on the other hand, became infected in due course (see Table C, p. 616).

Two days later (31 : vii), rat 368b was put into bell-jar A, rat 369a into bell-jar B, rat 370a into bell-jar C, and rat 371a into bell-jar D.

The next day (1 : viii), rats 369a, 370a and 371a were removed from the bell-jars B, C, and D, and the fleas on them carefully recovered and put back into their respective bell-jars. Rat 368b was left in bell-jar A. In the sequel rats 368b, 369a, and 370a did not become infected; rat 371a showed infection in due course.

Two days later (3 : viii), clean rats 369b, 370b and 371b were placed in bell-jars B, C, and D, and left in till they should become infected. Rat 369b did not become infected; rats 370b and 371b became infected in due course.

The results of the experiment may be summarised in the following manner. We start with four batches (A, B, C, D), each of thirty fleas, which had been exposed to infection on the same rat for one day (22 : vii to 23 : vii).

(1) Batch A (bell-jar A) :

Put on rat 368 from 25 : vii to 26 : vii (about three days after exposure to infection); result negative.

Put on rat 368a from 28 : vii to 29 : vii (about six days after exposure to infection); result negative.

Put on rat 368b, 31 : vii (about nine days after exposure to infection), and left on the rat; result negative.

This batch therefore did not become infective at all.

(2) Batch B (bell-jar B) :

Starved for five days (23 : vii to 28 : vii), then put on rat 369 from 28 : vii to 29 : vii (about six days after exposure to infection); result negative.

Put on rat 369a from 31 : vii to 1 : viii (about nine days after exposure to infection); result negative.

Left in with rat 369b on 3 : viii; result negative.

This batch therefore did not become infective at all.

(3) Batch C (bell-jar C) :

Starved for five days, then put on rat 370 from 28 : vii to 29 : vii (about six days after exposure to infection); result negative.

Put on rat 370a from 31 : vii to 1 : viii (about nine days after exposure to infection); result negative.

Left in with rat 370b on 3 : viii; result positive (the examinations of the rat indicate that infection took place between 4 : viii and 7 : viii).

This batch therefore became infective.

(4) Batch D (bell-jar D) :

Starved for five days, then put on rat 371 from 28 : vii to 29 : vii (about six days after exposure to infection) ; result positive.

Put on rat 371a from 31 : vii to 1 : viii (about nine days after exposure to infection) ; result positive.

Left in with rat 371a on 3 : viii ; result positive.

This batch evidently became strongly infective.

Remarks.—From the above summary it is seen that batch A (not starved) and batch B (starved) failed to become infective, while batches C and D (both starved) became infective.

Batch C did not produce its first infection before 3 : viii ; that is to say not until eleven or twelve days, at least, after exposure of the fleas to infection. It is not legitimate, however, to conclude from this that the developmental cycle of the trypanosome was retarded, since it has been shown above that infective fleas often fail to infect. The fleas may very well have been infective when placed in contact with rats 370 and 370a, but they were in contact with these rats for only about twenty-four hours. The most probable explanation for the two failures to infect is that only a small number of fleas in this batch were infective.

Batch D produced its first infection between 28 : vii and 29 : vii, and since it was exposed to infection between 22 : vii and 23 : vii it follows from these figures that the incubation-period—that is to say, the length of time taken by the developmental cycle of the trypanosomes—must have been between five and seven days. We are justified therefore in concluding that the trypanosomes in this batch went through a cycle of perfectly normal duration. The further fact that this batch never failed to produce infection during the time the experiment was carried on, indicates that the trypanosomes went through their cycle and established themselves successfully in a relatively large number of the fleas.

To conclude : Batches C and D show that starvation of the

fleas during the incubation-period does not inhibit the development of the trypanosomes; and batch D shows further that the development is not necessarily retarded by starvation.

(xix) Starvation of the Flea following immediately on an Infective Feed favours the Establishment of the Haptomonad Phase in the Rectum, while Starvation begun after the Incubation-Period in the Flea is over favours Migration to the Post-Pyloric End of the Intestine and the Establishment of the Haptomonad Phase there.

Experiments 49 and 50 were carried out with the object of ascertaining what effect, if any, varying food-conditions might have on the incidence and location of the established haptomonad phase in the flea's gut.

Experiment 49.—21 : iii : '14.—A number of fleas collected from the non-infected breeding-cage two days previously were put into a bell-jar with a well-infected rat at eight a.m., and were recovered again at twelve noon. They were then divided into two batches. Batch A, consisting of fifteen fleas, was put into a flask with moist sand at the bottom. Batch B (about forty fleas) was put into a bell-jar with a clean rat (rat 477).

26 : iii : '14.—Five fleas of batch A and four of batch B were dissected and examined.

Of batch A four were positive, one was negative. Of the four positive three showed developing forms of the trypanosomes in both the stomach and the rectum. Two of the three showed large numbers in both stomach and rectum, and in one of the stomachs intracellular forms were found. The fourth positive showed a haptomonad infection in the rectum.

Of batch B only one of the four dissected showed trypanosomes, and these were found free in the stomach-slide.

27 : iii : '14.—Eight fleas of batch A and eight of batch B were dissected and examined.

Of batch A seven were positive; one was negative. Of the seven positive one showed long active forms in the stomach and haptomonads in the rectum, while six showed developing forms in the rectum only—three scanty and three in fair numbers attached mostly round the

rectal surface of the projecting intestine, but in other parts as well. Of batch B only one was found infected, and it showed haptomonads attached about the middle region of the rectum.

The remaining fleas of batch B were now divided into two batches—batch A1 and batch B1. Batch A1, consisting of fifteen fleas, was put into a flask with moist sand at the bottom, and batch B1 was left in the bell-jar with rat 477.

4 : iv : '14.—Rat 477 was removed from the bell-jar and clean rat 478 was put in its place with batch B1.

Four fleas of batch A1 and four of batch B1 were dissected and examined.

Of batch A1 three were positive and one was negative. Of the three positive two showed trypanosomes in abundance in the post-pyloric region of the intestine and nowhere else. In these the trypanosomes were long and slender, and some were club-shaped; while in a third, which showed one or two in the rectum also, there was a swarming infection of haptomonads, as well as long, slender and club-shaped forms in the post-pyloric region. The fourth flea was negative.

Of batch B1 all were negative.

The remaining fleas of batch A1 were now allowed to feed on a clean rat for a short time.

9 : iv : '14.—Five fleas of batch A1 and four of batch B1 were dissected and examined.

Of batch A1 only one flea was positive, and it showed a fair number of slender trypanosomes in the post-pyloric region and nowhere else. Of batch B1 all were negative.

11 : iv : '14.—The remaining fleas of batch A1 were allowed to feed on a clean rat for a short time.

16 : iv : '14.—Five fleas of batch A1 and five of batch B1 were dissected and examined.

Of batch A1 two were positive and three were negative. The two positives showed large numbers of trypanosomes, some free, long and active, some club-shaped, and others were small, round and pear-shaped, in clumps and attached so as to form a lining to the wall of the gut. All were in the post-pyloric region and nowhere else. Of batch B1 all five were negative.

Rat 477 became infected in due course. Rat 478 never became infected. This agrees with results of the examinations.

Experiment 50.—20 : v : '14.—A number of fleas collected from the non-infected breeding-cage two days previously were put into a bell-jar with a well-infected rat late in the evening, were left overnight, and were recovered next morning. They were then divided into two batches. Batch A was kept in a flask with moist sand at the bottom.

Batch B was put into a freshly-prepared bell-jar with a clean rat (rat 496).

26 : v : '14.—Five fleas of batch A and five of batch B were dissected and examined.

Of batch A three were positive and two were negative. Of the three positive two showed haptomonads in the rectum, one being a swarming infection, and the third showed many long, active forms in the post-pyloric region only.

Of batch B all were negative.

28 : v : '14.—Seven fleas of batch A and seven of batch B were dissected and examined.

Of batch A five were positive and two were negative. Of the five positive four showed developing forms in the rectum only, and of these two were swarming infections. The fifth showed haptomonad infection of the rectum, and also free active forms in the stomach.

Of batch B only one of the seven was positive, and it showed a scanty infection of the rectum only.

The remaining fleas of batch B were now divided into two batches—batch A1 and batch B1. Batch A1 was put into a flask with moist sand in the bottom. Batch B1 was put into a bell-jar with a clean rat (rat 497).

2 : vi : '14.—Fourteen fleas of batch A1 and fourteen of batch B1 were dissected and examined.

Of batch A1 four were positive and ten were negative. Of the four positives one showed haptomonads on the rectal surface of the projecting intestine and three showed trypanosomes (one swarming) in the post-pyloric region and nowhere else.

Of batch B1 five were positive and nine were negative. Of the five positives one which had its stomach full of red blood showed a scanty infection in the rectum only. The other four showed infection in the post-pyloric regions only. Of these two (females) had small ova and their stomachs were empty. The remaining two had a fair quantity of brownish-coloured blood-débris in their stomachs. Rats 496 and 497 became infected in due course.

Summary.

A.—Fleas starved from immediately after the infective feed, dissected and examined five and six days after the infective feed.

No. of experiment.	Number of fleas examined.	Number infected.	Site of infection.	Remarks.
49	13	11	Stomach and rectum, 4; rectum only, 7	Intracellular forms in one stomach 3 scanty, 4 swarming —infections mostly in upper part of rectum
50	12	8	Stomach and rectum, 1; rectum only, 6; post-pyloric only, 1	3 scanty and 3 swarming

B.—Fleas that were put into bell-jar immediately after the infective feed, along with clean rat, on which they could feed at any time. Dissected and examined five and six days after the infective feed.

No. of experiment.	Number of fleas examined.	Number infected.	Site of infection.	Remarks.
49	12	2	Stomach only, 1	—
			Rectum only, 1	Haptomonad infection in middle region.
50	12	1	Rectum only, 1	—

A1.—Fleas in which starvation was begun six days after the infective feed. During the six days the fleas had been fed on clean rats. Those of Experiment 49 were dissected and examined fourteen, nineteen, and twenty-six days after the infective feed. Those of Experiment 50 were dissected and examined thirteen days after the infective feed.

No. of experiment.	Number of fleas examined.	Number infected.	Site of infection.	Remarks.
49	14	6	Post-pyloric only, 5; post-pyloric and rectum, 1	Swarming haptomonad infection in 2.
50	14	4	Rectum only, 1; post-pyloric only, 3	Pile carpet infection upper part; swarming haptomonad infection in 1.

B1.—Fleas kept with clean rats in bell-jar ever since the infective feed. Dissected and examined as in A1.

No. of experiment.	Number of fleas examined.	Number infected.	Site of infection.	Remarks.
49	13	0	—	—
50	14	5	Rectum only, 1; post-pyloric only, 4	Stomach full of red blood; [1]stomach empty and ova small in 2; stomach contained fair quantity of blood-débris in 2.

These results seem to throw light on the important function that the nectomonad forms described as occurring in the established rectal-phase may have in maintaining the infection in the flea. The optimum food-conditions for the establishment of the haptomonad stage seem to lie somewhere between abundance and poverty, and between partial and complete digestion of the blood-supply. More extended

[1] Although the fleas of batches B and B1 in both experiments had the chance of feeding on clean rats at any time from immediately after the infective feed onwards, all may not have equally availed themselves of the opportunity. It is certain, in fact, from the condition of the ova and of the stomachs of two females of batch B1 that showed post-pyloric infection, that they, for some reason not ascertained, had starved in the midst of plenty; and these should really be transferred to batch A1. The remaining two fleas of batch B1 that showed post-pyloric infection had evidently fed, but not quite recently. The other infected flea of batch B1 had its stomach distended with red blood, and in it the infection was in the rectum only.

In order to test the infectivity of batches A1 and B1 in Experiment 50, twelve fleas of each batch were put on clean rats, two fleas to each clean rat. The result was that two of the six rats belonging to batch A1 became infected, while none of the six belonging to batch B1 became infected. This may indicate that a period of starvation heightens the infectivity of infected fleas, perhaps by inducing increased production of the final propagative forms of the cycle; but further experiments would be required to justify such a deduction.

and varied observations are required, but so far as these experiments go they show that the incidence, location, and continued existence of the haptomonad stage in the flea's gut depend to a large extent on the food-supply. When, under conditions of partial starvation, a sufficient supply of nourishment cannot be obtained in the rectum, the haptomonad stage, if established there, would die out, and the flea would lose its infection were it not that the nectomonads produced in the rectum migrate forwards and re-establish this stage nearer to the food-supply. In like manner it may be assumed that when the food-supply in the post-pyloric end of the intestine becomes continuously too rich and abundant, the nectomonads produced there migrate backwards to the rectum and so the balance is maintained and the infection in the flea is kept up.

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